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MICROBIAL ECOLOGY MEASUREMENT SYSTEM

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FINAL REPORT  
FOR PERIOD OCTOBER 1970 THROUGH JULY 1972

To  
NASA/MANNED SPACECRAFT CENTER  
Preventive Medicine Office  
Houston, Texas  
77058

Contract No. NAS 9-11371

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**AEROJET MEDICAL AND  
BIOLOGICAL SYSTEMS**

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## SECTION I

1.0

### SUMMARY

The original study for development of techniques and reagents for assessment of viral ecology used influenza A<sub>2</sub> (Hong Kong variety), Adenovirus type 4, Herpesvirus, Echovirus type 9, Coxsackievirus type A<sub>2</sub> and Mycoplasma pneumoniae. These organisms were used to prove the feasibility of the Passive Immune Agglutination (PIA) System (see Final Report Contract No. NAS 9-9740).

The sensitivity and potential rapidity of the PIA test that was demonstrated during the feasibility study warranted continuing the effort to examine the possibility of adapting this test to an automated procedure that could be used during manned missions. The effort was continued under Contract NAS 911731 during which time many advancements over the original system were made. The effort during this program has optimized the test conditions for two important respiratory pathogens, influenza virus and Mycoplasma pneumoniae, developed a laboratory model automated detection system, and investigated a group antigen concept for virus detection.

The manual PIA test has a high degree of technician involvement, therefore several devices were evaluated for the automation of this test. The sensor devices chosen were the Celloscope conductometric system and the photometric system, both of which greatly facilitated quantitating of the PIA reaction. The photometric system was selected by NASA for further study and a test bed was built and tested. The results obtained were compared with those from the celloscope.

The sensitivity of the automated PIA test was determined for influenza to be 0.128 hemagglutinating units per ml (HA units/ml) and for M. pneumoniae to be about  $10^2$  to  $10^4$  colony forming units per ml (CFU/ml). Sensitivity for the mycoplasma system was not as good as hoped and varied from test to test. Recent developments in research on the immunology of M. pneumoniae might be used to alleviate this problem.

The use of the automated PIA reaction for bacterial detection was evaluated. Group A Streptococcus was detected with this system and although sensitivity was not good, feasibility was demonstrated. When optimum conditions are determined and the proper (precipitating) antibody used, sensitivity would be increased to an acceptable level.

A group antigen for the adenoviruses was isolated and antibody was produced in rabbits. The adenovirus group antibody reacted with other adenovirus types in immunodiffusion tests, complement fixation tests and in PIA tests where latex beads were sensitized with the antibody. Sensitivity and optimum conditions for the adenovirus group system could not be determined in the limited time available.

Preliminary tests on the handling of oropharyngeal clinical samples for PIA testing were performed using the adenovirus system. The results obtained indicated that the PIA signal is reduced in positive samples and is increased in negative samples. Treatment with cysteine appeared to reduce nonspecific agglutination in negative samples but did not maintain the signal in positive samples. The need for a more complete study on this problem is evident.

## SECTION 2

2.0

### INTRODUCTION

With the imminent development of anti-viral agents there is an increasing need for the rapid diagnosis of viral diseases. Present methods of virus isolation and identification commonly involve the use of tissue cultures in order to increase the virus population for use in subsequent tests for identification. A minimum of 72 hours and a maximum of upwards to one month are necessary to accomplish the isolation procedures. Identification of the particular virus in question may involve two to 24 hours for the more rapid tests such as complement fixation and hemagglutination inhibition to 2 - 7 days for neutralization tests which use specific antisera to inhibit the virus' ability to infect laboratory animals or susceptible tissue cultures. Using these methods the patient is usually either well or dead by the time identification is made in the laboratory. Even this type of virus identification is useful for epidemiological studies and to let physicians know what virus diseases to expect in their particular area.

Another method of virus identification relies upon an increase in the level of specific antibodies in the patient's serum. Since antibodies are not produced at a significant rate until 2 - 4 weeks after infection, this method does not give any guidance for treatment but is useful only for epidemiological purposes.

Rapid diagnosis of viral diseases will also help to eliminate the indiscriminate prophylactic use of antibiotics which have no effect on viruses but may have a detrimental effect on the patient. This is important whether there are anti-viral agents available or not.

The need for early and rapid diagnosis of viral diseases becomes most evident in long term space flights. The confined quarters which are found on space crafts provide excellent conditions for the rapid

spread of viral and other diseases. If an efficient and rapid method of detection were available, one which could detect viruses in an astronaut before the symptoms of the disease were evident, the afflicted person could be isolated from the rest and/or treated if treatment were available. With this knowledge the space flight could be aborted, if the disease were serious, before all personnel were incapacitated to an extent that return would be impossible.

The use of a rapid method of detecting viruses early in the course of disease would be invaluable when used before the space flight took place. In this case the affected astronauts could be replaced.

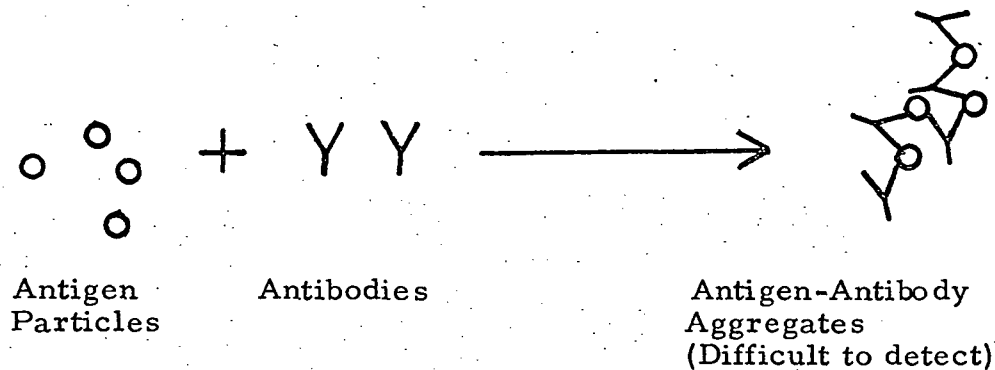
Such a method of viral detection is being researched at AMB. It is called the Microbial Ecology Monitoring System (MEMS) and utilizes the passive immune agglutination (PIA) technique. When viruses and certain types of homologous antibodies react, the viruses are agglutinated; however, this agglutination is submicroscopic and therefore undetectable if the viruses are present in low numbers. In the PIA technique, latex spheres (beads), on the order of 1 to 2  $\mu\text{m}$  in size, are coated with specific antibody. When they react with viruses, agglutination occurs but this agglutination is detectable because of amplification by the beads. This agglutination can be seen microscopically and even macroscopically if the antigen is present in high enough numbers. A diagrammatic representation of agglutination is given in Figure 1.

Bead agglutination tests are in common usage to detect antigen-antibody reactions. In some the antigen is attached to the beads to detect antibody and in others antibody is coated on the beads and will detect antigen. Such tests are available commercially to detect rheumatoid factor, C-reactive protein, antinucleoprotein factors associated with systemic lupus erythematosus, and fibrinogen levels among others. In these tests the patients specimen is reacted with the sensitized beads and macroscopic agglutination usually indicates a positive test.

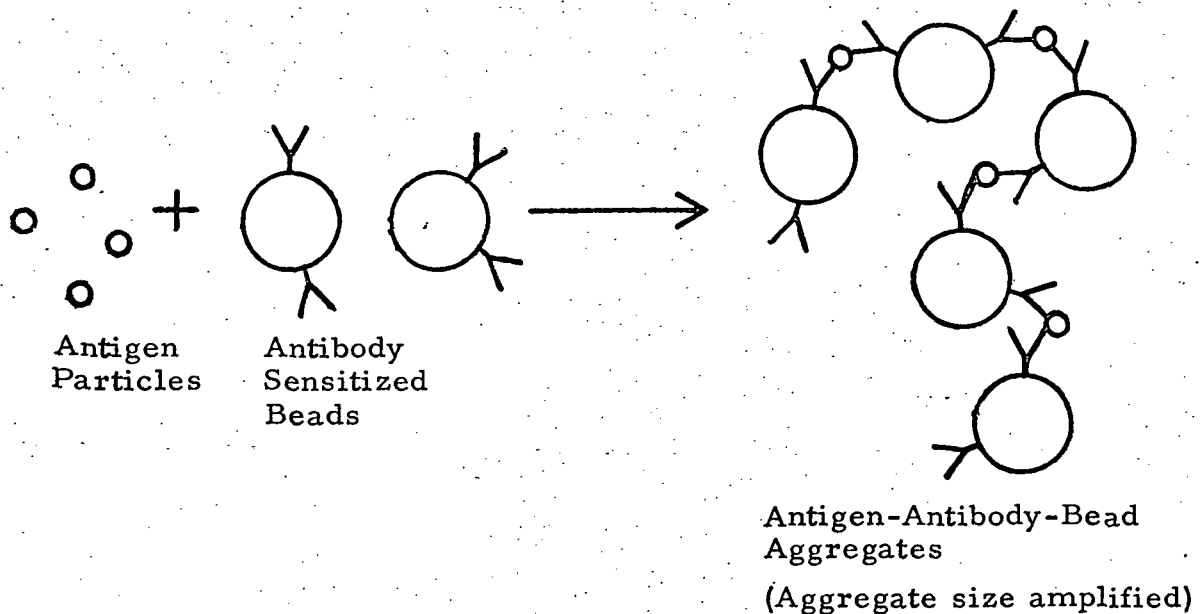


FIGURE 1  
 DIAGRAMATIC COMPARISON OF DIRECT VERSUS BEAD  
 AMPLIFIED IMMUNE AGGLUTINATION

a. Agglutination of Antigens by specific antibodies



b. Agglutination of antigens by specific antibodies attached to beads (PIA test)



Detection of agglutination when using the PIA approach is accomplished manually by microscopic examination and counting of the various size clumps or automatically by either measuring differences in conductances of the clumps or differences in the transmission of light through an agglutinated sample. Very small amounts of antigen are detectable using this method and the time involved in completing a test is about two hours.

The organisms used in the present study were Influenza A<sub>2</sub>, Hong Kong, which is a common respiratory virus and Mycoplasma pneumoniae, a respiratory pathogen most commonly found in children, young adults and military recruits. It is not a virus but is much smaller than bacteria and can grow in artificial media. Viruses, such as influenza, must have a living host such as the embryonated hen's egg, various animals, or tissue cultures in which to multiply. These organisms were chosen for study because they represent respiratory pathogens from two entirely different groups of microorganisms, viruses and mycoplasmas.

Another part of the study was devoted to the use of the PIA for multiagent detection. In this case an antigen common to all adenoviruses was isolated, antibody produced to it, beads sensitized and tested for their ability to detect heterologous adenovirus.

The study reported here is divided into three parts. Section 3 includes the optimization of PIA sensitivity, specificity and reliability utilizing influenza and M. pneumoniae. Section 4 is devoted to breadboard design, fabrication and evaluation utilizing the photometric approach. Section 5 entails studies involving the detection of bacteria by PIA along with the use of a group antigen to detect heterologous adenovirus types.

## SECTION 3

### 3.0 OPTIMIZATION OF PIA SENSITIVITY, SPECIFICITY AND RELIABILITY

The optimization of sensitivity, specificity and sensitivity of the PIA test utilized two organism systems. Influenza A<sub>2</sub> was chosen as a typical virus system whereas Mycoplasma pneumoniae represented the mycoplasma species.

Optimization of the PIA test involves optimization of reagents and preparation methods, particularly preparation of sensitized beads, and optimization of the conduct of the test itself.

#### 3.1 REAGENT PRODUCTION

##### 3.1.1 ANTIGEN PRODUCTION

##### 3.1.1.1 Influenza A<sub>2</sub>

The virus used in this portion of the program was Influenza A<sub>2</sub> (Hong Kong, 1968). High titered virus suspensions were produced and the antigen used for immunization of animals was devoid of any detectable host protein. The virus was propagated in the allantoic cavity of 10 - 11 day old embryonated hen's eggs by injecting 100 EID<sub>50</sub> of seed influenza virus in 0.1 ml of Hank's Balanced Salt Solution (HBSS). The HBSS contained 50 units each of penicillin and streptomycin per ml along with 2.5 µg per ml of amphotericin B. The virus was injected with a syringe through a hole punch in the shell 3 - 5 mm above the air sac membrane. The holes were sealed with transparent tape and the eggs incubated for 48 hours at 35 C. After incubation, the eggs were placed at 4 C overnight to reduce bleeding during harvesting. The chorioallantoic fluid (CAF) was then withdrawn with a 10 cc syringe and needle and the fluids from the eggs were pooled, clarified by centrifugation at 4000 x g for 10 minutes and titered by hemagglutination (HA) using chicken erythrocytes.

The virus was partially purified by barium sulfate adsorption and elution with citrate buffer in the following manner: To one liter of CAF virus, 50 grams of barium sulfate was added along with potassium oxalate to 0.16 M. The mixture was stirred for 90 minutes at 4 C and allowed to stand overnight (the virus is adsorbed to the barium sulfate). The barium sulfate adsorbed virus was then centrifuged at 4000 x g for 5 minutes and the supernatant fluid discarded. The virus was eluted from the barium sulfate in concentrated form by the addition of one-seventh the original CAF volume of citrate buffer composed of 0.25 M sodium citrate, 0.1 M NaCl, 0.4 M Tris, 0.16% Tween 80 and 0.2% gelatin, pH 7.2. This mixture was allowed to stand overnight at 4 C at which time the barium sulfate was removed from the resuspended virus by centrifugation at 800 x g for 10 minutes.

The partially purified virus was purified and concentrated further by ultracentrifugation on a sucrose cushion. Ten ml of 60% sucrose (w/w) in 0.01 M phosphate buffered saline (PBS) containing 0.02% gelatin was placed in a 60 ml cellulose nitrate centrifuge tube. Ten ml of 30% sucrose (w/w) in the same buffer was carefully layered on top. Forty ml of the citrate virus eluate was then layered on top of the 30% sucrose and the tubes (3) were centrifuged for 3 hours at 106,000 x g using the SW 25.2 rotor in the Beckman L2 ultracentrifuge. After centrifugation, the virus was collected from the interface of the two sucrose solutions. The sucrose was removed by dialysis against PBS overnight and the virus inactivated with formalin (1:1000) and preserved with 0.01% merthiolate. This purified influenza virus was then checked for purity by gel diffusion against anti-chicken allantoic fluid serum and titered by HA. Using this method of purification, no precipitin lines were detected in gel diffusion tests and no antibodies to CAF were produced in animals immunized with this antigen.

The PIA influenza test antigen was produced in the same manner in embryonated eggs but was not purified. The CAF virus harvested was, however, pooled and titered for EID<sub>50</sub> per ml and for HA.

### 3.1.1.2 Mycoplasma pneumoniae

Mycoplasma pneumoniae for use in immunizing animals was grown in a medium consisting per liter of: 700 ml PPLO Broth (Difco), 200 ml agamma horse serum (Microbiological Associates), 100 ml of 25% yeast extract (Microbiological Associates), 0.05% thallium acetate and 1000 units per ml of penicillin. The organisms were grown in 1000 ml Erlenmeyer flasks containing 500 ml of the above broth medium. Ten ml of seed containing about  $10^7$  colony forming units (CFU) per ml was inoculated into each flask and incubated seven days at 37C. The flasks were shaken once daily and on the seventh day were harvested and concentrated by centrifugation at about 25,000 x g in the GSA rotor in a Sorval RC2-B refrigerated centrifuge. The pelleted mycoplasma were suspended in PBS, washed three times, and resuspended in PBS at 1/500 the original volume. Typically, CFU titers of concentrated M. pneumoniae harvests were  $10^{10}$  CFU/ml, however, since the organism grows in aggregates of individual cells plus the fact that centrifugation causes additional aggregation, the actual titers were probably much higher.

Complete removal of medium components, especially agamma horse serum, could not be accomplished and antisera to the M. pneumoniae demonstrated precipitin lines when tested against medium components in gel diffusion tests. This necessitated the production of M. pneumoniae on the glass surfaces of two-liter Provitsky bottles in 200 ml of a medium consisting of 10.5g PPLO Broth (Difco), 40 ml of 25% fresh yeast extract (Microbiological Associates), 4.8g powdered Eagle's Minimal Essential Medium (GIBCO), 20 ml of PPLO serum fraction (Difco), 5g glucose, 20 mg phenol red, 11.9 g HEPES buffer (Calbiochem) and oleic acid (1.5 mg/ml in 95% ethanol added to a final concentration of 6 µg/ml of medium). Distilled water was added to bring the volume to 1000 ml.

The bottles were inoculated with 4 ml of M. pneumoniae ( $10^6$  CFU/ml) which had previously been adapted to the medium. The bottles were stoppered and incubated at 37 C for 2-3 days until a confluent monolayer of growth was observed. The organisms were harvested by decanting off the medium, washing 4 times with sterile PBS, then scraping the monolayer from the glass with a rubber policeman. The cells were

suspended in 20 ml of PBS titered for CFU, aliquoted and frozen at -70C until used.

### 3.1.2 ANTIBODY PRODUCTION

#### 3.1.2.1 Production of Antisera

Hyperimmune sera were produced in New Zealand white rabbits and goats from both the influenza and M. pneumoniae antigens.

Rabbits were usually given four weekly injections of one ml intramuscularly and exsanguinated seven days after the final injection after testing a trial bleeding for antibodies. Goats were also given weekly one ml intramuscular injections and blood (about 300 ml) was withdrawn at monthly intervals. The sera were separated from the clots and tested for antibodies.

Influenza antibodies were tested by the usual hemagglutination inhibition (HAI) test using receptor destroying enzyme to remove non-specific inhibitors of agglutination and four HA units of CAF influenza as the antigen. Typically HAI titers of 1:640 to 1:2560 were obtained using this injection schedule. Tests for purity were performed by gel diffusion against concentrated CAF. No anti-CAF antibodies were found in sera from animals immunized with purified influenza antigens.

Agglutination titers from sera obtained from animals immunized with M. pneumoniae could not be determined, although many types of tests were tried, because of interference from agamma horse serum antibodies. Sera were determined acceptable, however, when tested by gel diffusion against glass grown M. pneumoniae which had been subjected to six freeze-thaw cycles to release soluble antigenic components and an agamma horse serum control. Previous tests had demonstrated no antibodies to the other medium components.

Usually a precipitin line formed against the mycoplasma which was not in identity with any lines formed against agamma horse serum. If this was the case, the serum was judged as satisfactory.

### 3.1.2.2 Antibody Purification

Immunoglobulins (IgG) were isolated from the serum by ammonium sulfate precipitation at 4 C and pH 7.8. Equal volumes of serum and distilled water were combined. A volume of saturated ammonium sulfate equal to the combined volumes of serum and water was added dropwise with constant stirring. The pH was adjusted to 7.8 with 0.1 M NaOH and the precipitate was allowed to stir overnight at 4°C. The next day, the precipitate was centrifuged at 5000 x g for twenty minutes and the supernatant fluid discarded. The precipitate was washed once with 40% saturated ammonium sulfate and then redissolved with phosphate buffered saline (PBS) to the original serum volume. To this solution one-half volume of saturated ammonium sulfate was added dropwise while stirring as before. The pH was again adjusted to 7.8 and the precipitate allowed to stir at 4 C for two hours. After centrifugation, the precipitate was redissolved to one-half the original serum volume with PBS and the ammonium sulfate removed by percolating the IgG solution through a column of G-25 Sephadex. Fractions were collected and tested qualitatively for protein. Those fractions containing protein were combined, assayed for protein quantities, and stored frozen at -70 C.

### 3.1.3 PREPARATION OF SENSITIZED LATEX BEADS

During the course of the program beads of various sizes and various compositions were used. However, in all cases essentially the same method of bead sensitization was used.

The stock suspension (10%) of beads was washed in distilled water and resuspended in 10 volumes of glycine buffered saline, pH 9.0 (GBS). A volume of antibody in GBS equivalent to the original volume of stock beads, ranging in concentration from 1.25 to 5.0 mg/ml of antibody protein depending on bead size. The mixture was agitated gently for 30 minutes at room temperature after which an equal volume of GBS was added and the incubation was continued for an additional 30 minutes.

The supernatant was removed after centrifugation at 3000 x g for 5 minutes. The resultant beads were reconstituted to 25 times original volume in GBS containing 0.2% crystalline bovine albumin.

Studies were performed on  $2.0\ \mu$  size beads which had been sensitized with either influenza or mycoplasma antibody to determine the effect of storage on bead reactivity. The beads were stored at  $4^{\circ}\text{C}$  and tested periodically, for up to sixteen weeks with the influenza beads and nine weeks with the mycoplasma beads. Reactivity was tested against dilutions of standard antigens in the Celloscope. The data are shown in Tables 1 and 2. The results are given as the log of the signal to noise ratio ( $\log S/N$ ) and are graphically demonstrated in Figures 2 and 3. The data show excellent stability of the bead preparations over the periods of time they were stored.

TABLE 1

STABILITY OF  $2.0\ \mu$  SIZE BEADS SENSITIZED WITH  
M. PNEUMONIAE ANTIBODY STORED AT  $4^{\circ}\text{C}$

| <u>M. pneumoniae</u><br>Concentration<br>CFU/ml | Log S/N <sup>1</sup> |         |
|---|----------------------|---------|
|   | 0 Weeks              | 9 Weeks |
| $5 \times 10^4$                                 | 0.18                 | 0.08    |
| $1 \times 10^5$                                 | 0.49                 | 0.45    |
| $5 \times 10^5$                                 | 1.13                 | 1.66    |
| $1 \times 10^6$                                 | 1.33                 | 2.00    |
| $5 \times 10^6$                                 | 1.83                 | --      |

<sup>1</sup>S/N = Signal to noise ratio

TABLE 2

STABILITY OF  $2.0\ \mu$  SIZE BEADS SENSITIZED WITH  
INFLUENZA ANTIBODY STORED AT  $4^{\circ}\text{C}$

| Influenza Antigen<br>Concentration<br>HA Units/ml | Log S/N |          |          |
|---|---------|----------|----------|
|   | 0 Weeks | 12 Weeks | 16 Weeks |
| .013  | 0.01    | -0.11    | --       |
| .026  | --      | --       | 0.04     |
| .128  | 0.10    | 0.20     | 0.16     |
| .256  | --      | --       | 0.26     |
| 1.28  | 1.59    | 1.01     | 1.14     |
| 2.56  | --      | --       | 1.68     |
| 12.8  | 2.48    | 2.02     | 2.19     |



FIGURE 2  
STABILITY OF SENSITIZED 2.0  $\mu$   
LATEX BEADS - M. PNEUMONIAE

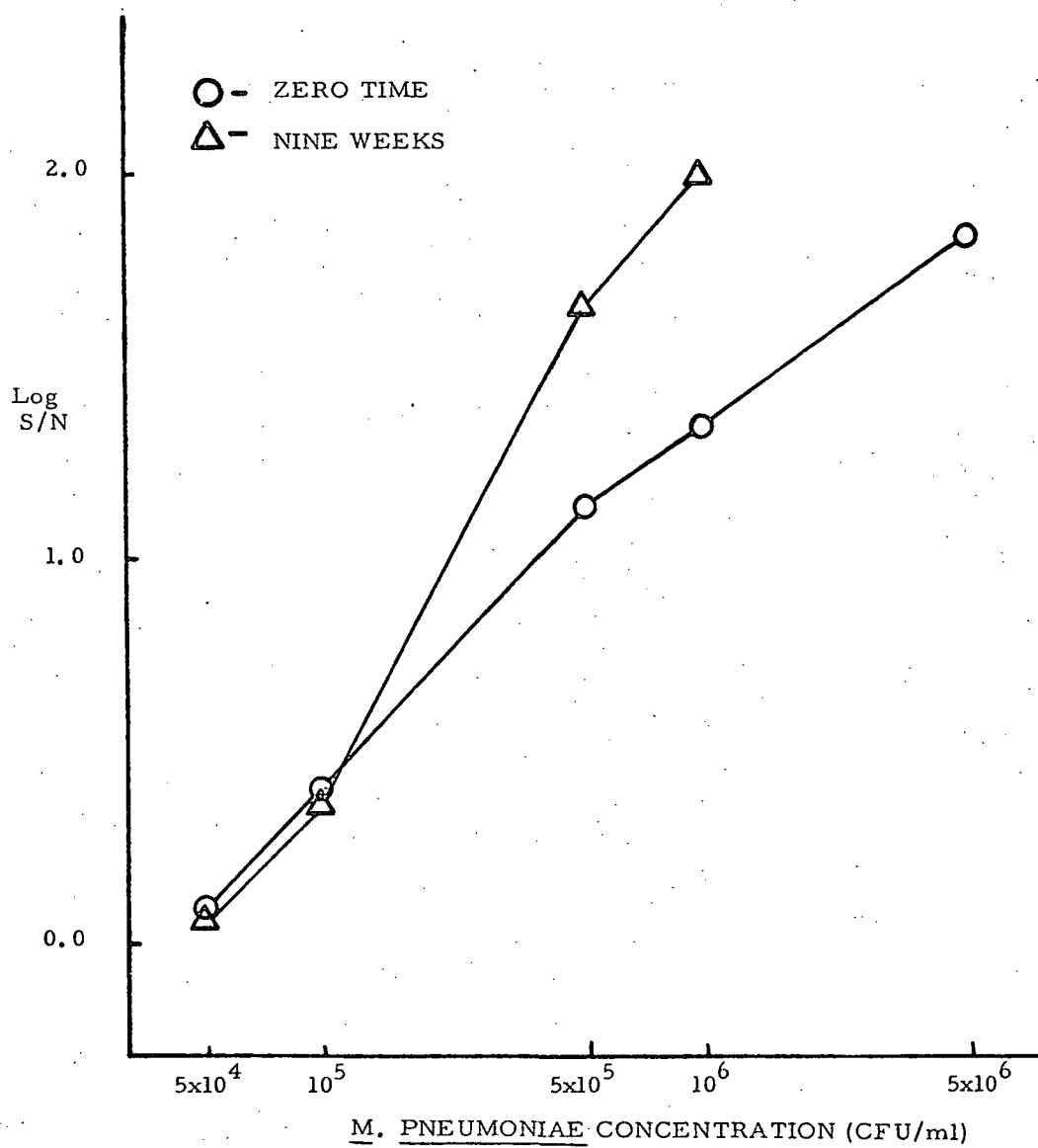
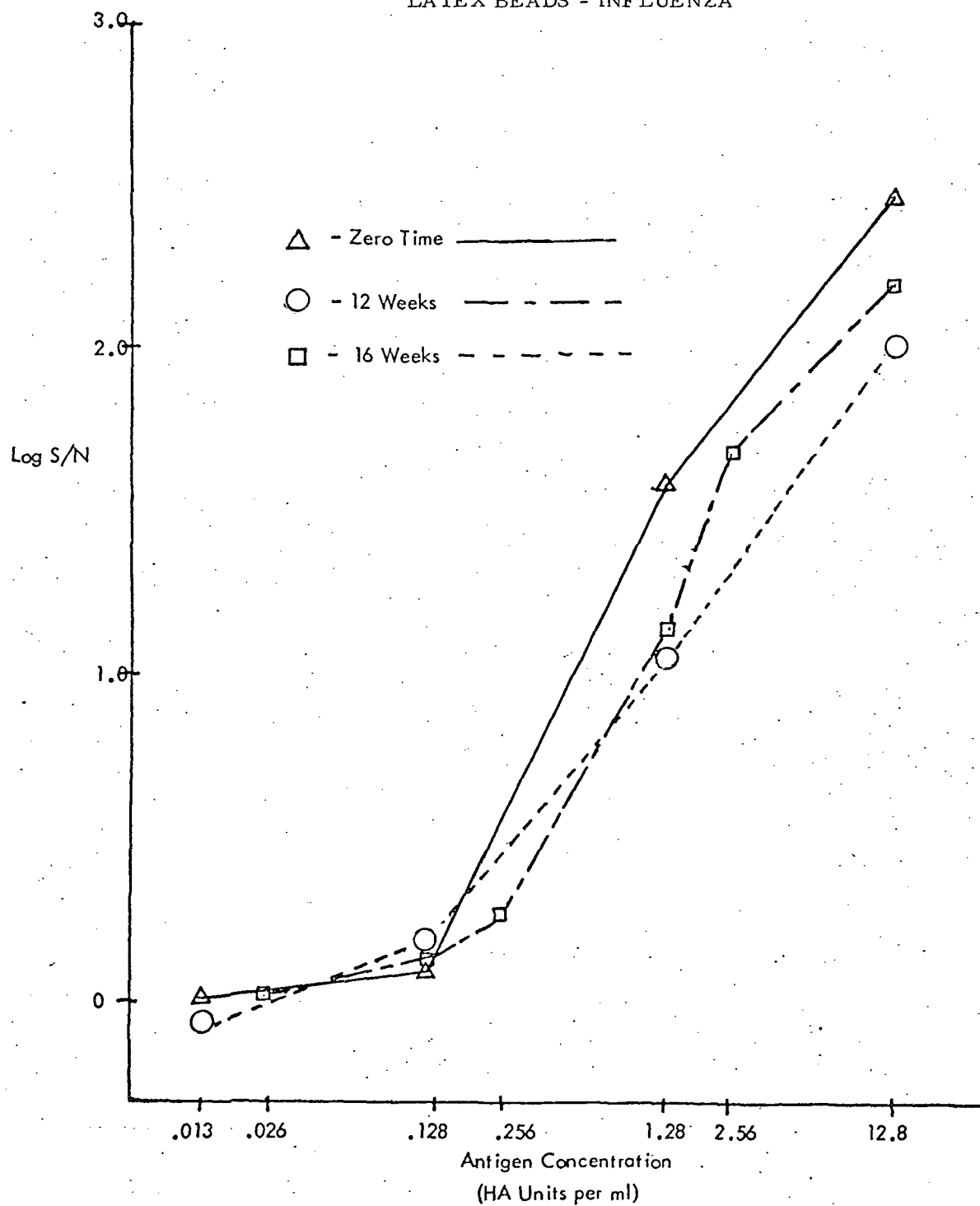


FIGURE 3  
STABILITY OF SENSITIZED  $2.0\mu$   
LATEX BEADS - INFLUENZA



## 3.2 OPTIMIZATION OF REACTION CONDITION VARIABLES

### 3.2.1 BEAD SIZE

The most critical reagent of the PIA test is the sensitized latex bead. During this project it became apparent that the optimum bead size depended on the type of readout used; e. g., manual PIA, Celloscope (discussed later) or photometer (also discussed later).

To determine optimum bead size, beads of various sizes (0.22 - 3.50  $\mu$  in diameter) were sensitized with anti-influenza IgG at a final concentration of 0.05 mg protein per ml (1.25 mg protein per ml of stock beads). The sensitized bead preparations were reacted under standard PIA conditions with a standard influenza antigen ( $10^2$  EID<sub>50</sub> per ml). Bead composition was varied because polystyrene latex was not commercially available in the 2.0 and 3.5  $\mu$  sizes. The results, shown in Table 3, showed a significant difference from control with the 2.0  $\mu$  size particles and less difference with 1.1  $\mu$  particles. The other beads showed differences that were within the noise levels.

TABLE 3  
EFFECT OF BEAD SIZE ON MANUAL PIA SENSITIVITY

| BEAD SIZE<br>( $\mu$ Diameter) | BEAD COMPOSITION               | MEAN CLUMP SIZE <sup>1</sup><br>(Signal minus Noise) |
|--------------------------------|--------------------------------|--|
| 0.2                            | polystyrene latex              | 0.00   |
| 0.5                            | polystyrene latex              | 0.05   |
| 0.8                            | polystyrene latex              | 0.04   |
| 1.1                            | polystyrene latex              | 0.19   |
| 2.0                            | polyvinyltoluene               | 0.33   |
| 3.5                            | polystyrene divinyl<br>benzene | 0.00   |

<sup>1</sup> Average of three separate determinations.

2.0  $\mu$  size beads were also selected for use in the Celloscope apparatus. The Celloscope method of readout will be discussed later but briefly instead of signal minus noise (S-N), signal to noise ratios (S/N) were used. Using 2.0  $\mu$  beads, greater S/N ratios were found than with 1.1  $\mu$  size beads at the lower concentration when testing influenza antigen under the standard conditions. These data are shown in Table 4. Therefore, 2.0  $\mu$  polyvinyltoluene beads were used when testing with the Celloscope.

TABLE 4  
COMPARISON OF 1.1  $\mu$  AND 2.0  $\mu$  SIZE BEADS AS TO SIGNAL  
TO NOISE RATIOS (S/N) USING THE CELLOSCOPE  
TO MEASURE INFLUENZA ANTIGEN

| ANTIGEN<br>CONCENTRATION<br>EID <sub>50</sub> /ml | 1.1 $\mu$ BEADS |         | 2.0 $\mu$ BEADS |         |
|---|-----------------|---------|-----------------|---------|
|   | Log Signal      | Log S/N | Log Signal      | Log S/N |
| $5 \times 10^3$                                   | 11.023          | -.004   | 11.669          | .150    |
| $1 \times 10^4$                                   | 11.092          | .067    | 12.079          | .560    |
| $5 \times 10^4$                                   | 11.236          | .211    | 12.457          | .938    |
| $1 \times 10^5$                                   | 11.772          | .747    | 12.762          | 1.243   |
| $5 \times 10^5$                                   | 12.326          | 1.301   | 12.867          | 1.348   |
| $1 \times 10^6$                                   | 12.363          | 1.338   | 12.857          | 1.338   |
| Control   | 11.025          |         |                 |         |

Bead sizes for use in the photometric sensors were limited to those .8  $\mu$  or larger since smaller beads would have required the use of quartz cuvettes and a U. V. light source in order to measure peak absorbance. Beads .8  $\mu$  to 2.0  $\mu$  in size can be measured with visible light. Since most of the previous work had been done on 1.1  $\mu$  and 2.0  $\mu$  size beads, they were compared in tests to detect influenza antigen at the wavelengths of light which were ideal for each size bead. The results of

this comparison are given in Figure 4. As can be seen, the 1.1  $\mu$  beads showed a somewhat increased sensitivity and higher signal than did the 2.0  $\mu$  size beads so they were used with the photometer.

The optimum conditions for sensitizing 2.0  $\mu$  beads with IgG was with 1.25 mg protein per ml of stock beads and 3-5 mg protein per ml of stock 1.1  $\mu$  size beads. With both sizes sensitization took place at room temperature at a 1:25 dilution for thirty minutes while mixing gently. A higher concentration of protein was necessary because of the increased surface area of 1.1  $\mu$  size beads.

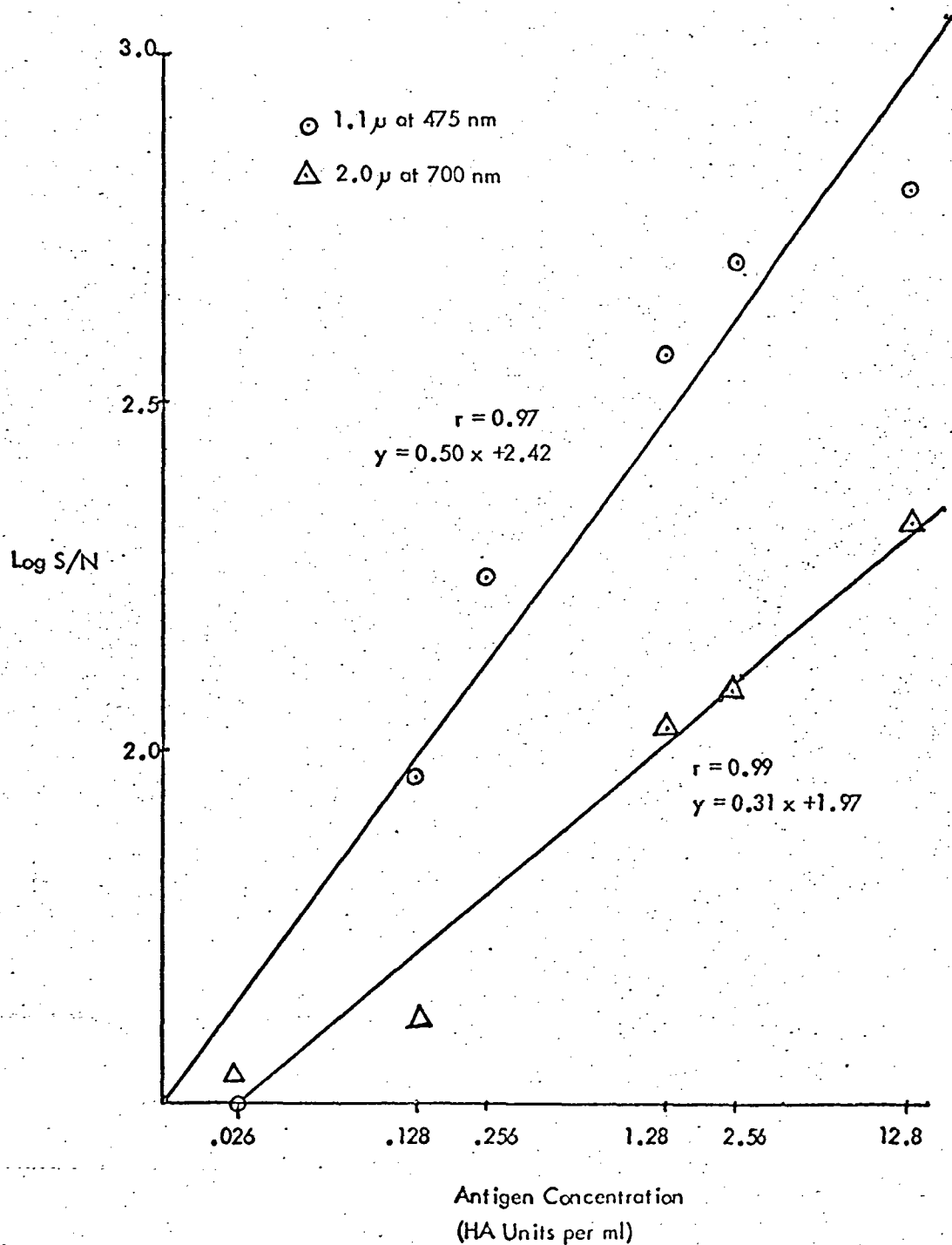
### 3.2.2 IONIC STRENGTH AND pH

For both sensitization of beads with antibody and PIA testing, a buffer consisting of 0.1 M glycine and 0.05 M NaCl (GBS) at pH 9.0 was selected. All sensitization of beads and PIA testing was performed in this buffer. In addition, occasionally 0.2% crystalline bovine serum albumin was added to GBS to store sensitized bead suspensions to minimize non-specific clumping.

### 3.2.3 TIME AND TEMPERATURE REACTION CONDITIONS

Three temperature reaction conditions were evaluated for improving PIA sensitivity, 25 C, 40 C and 50 C. These temperatures were selected because most serological reactions proceed to completion throughout the range of 25-50 C. Both the influenza and mycoplasma systems were tested in this manner. Samples were taken periodically up to 60 minutes with the mycoplasma and 90 minutes with influenza. The antigen concentration for the influenza system was  $10^2$  EID<sub>50</sub>/ml and for the mycoplasma  $10^2$  CFU/ml. The data for these experiments are given in Table 5. As can be seen, the results at 50 C were erratic for both systems. Maximum response with the influenza system was attained after 60 minutes incubation at a temperature of 40 C. With the mycoplasma system, however, the highest response at 40 C was reached

FIGURE 4  
 BEAD SIZE COMPARISON — PHOTOMETER  
 MYXOVIRUS SENSITIZED 1.1 $\mu$  AND 2.0 $\mu$  LATEX BEADS



after 30 minutes incubation but an even greater MCS (S-N) value was attained at an incubation temperature of 25 C (room temperature). This response increased over all the time intervals studied. A reaction temperature of 40 C was chosen for both systems with 60 minutes incubation for influenza and 30 minutes for the mycoplasma.

TABLE 5

COMPARISON OF VARIOUS INCUBATION TIMES  
AND TEMPERATURES ON THE PIA REACTION

| Incubation Time<br>(min.) | MEAN CLUMP SIZE (Signal Minus Noise) |      |      |                      |      |      |
|---------------------------|--------------------------------------|------|------|----------------------|------|------|
|                           | INFLUENZA                            |      |      | <u>M. PNEUMONIAE</u> |      |      |
|                           | 25 C                                 | 40 C | 50 C | 25 C                 | 40 C | 50 C |
| 15                        | 0.24                                 | 0.21 | 0.50 | 0.08                 | 0.24 | 0.35 |
| 30                        | 0.33                                 | 0.28 | 0.31 | 0.43                 | 0.37 | 0.26 |
| 45                        | -                                    | -    | -    | 0.52                 | 0.39 | 0.34 |
| 60                        | 0.34                                 | 0.57 | 0.53 | 1.17                 | 0.36 | 0.25 |
| 90                        | 0.52                                 | 0.66 | 0.03 | -                    | -    | -    |

### 3.3 READOUT SELECTION

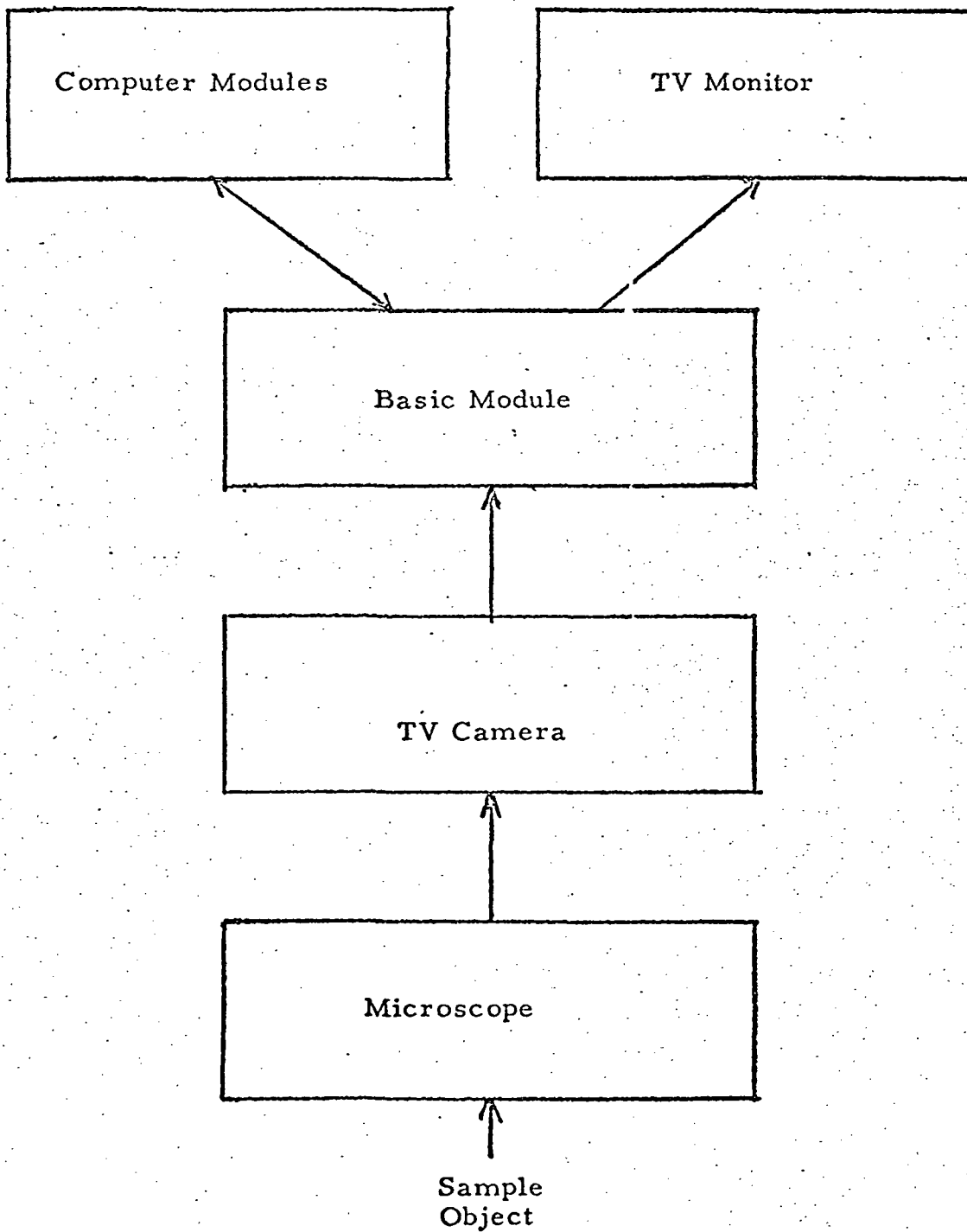
Several different types of sensors to automate the PIA test were evaluated. They were: (1) Millipore Particle Measurement System; (2) Royco Particle sensor; (3) Hiac Particle sensor; (4) Electrozone/Celloscope Particle Sensor; and (5) a device which would measure particle size by light scattering which was represented by the Beckman DK-2 Spectrophotometer.

#### 3.3.1 MILLIPORE PARTICLE MEASUREMENT COMPUTER SYSTEM

##### 3.3.1.1 System Description

The Millipore Particle Measurement Computer System (MPMC) is an electro-optical system used for the determination of particle sizes or distributions (or other such parameters) by quantitative image analysis. The system is composed of a microscope (Bausch and Lomb Flatfield Dynoptic), a television camera, a television monitor, specific computer modules for data gathering and analysis, and a control box (see Figure 5).

FIGURE 5  
MILLIPORE SCHEMATIC





Briefly, a microscope slide with the sample preparation is placed on the microscope stage, and its image is detected by the television camera. The light from the microscope (bright field or darkfield) is detected by the camera. The basic module accepts the video signal from the camera and processes it into digital form. This signal is then passed to the other computer modules for data processing and returned to the basic module, to be displayed on the T. V. monitor.

Several of the computer modules were evaluated for quantitating the PIA reaction. The first sensor subsystem was comprised of the "over-sized count and entire field measurement" module used to size an entire field of particles by total length or total area. By coupling the "entire field count" module to this subsystem, it is possible to obtain the following additional parameters: average area per clump, average projected length per clump, and the total count. In the second sensor subsystem, the "selective particle measurement" module was used for measuring the area, the projected length of Feret's diameter of individual particles, through the use of a light pen. When used in conjunction with the "size distribution" module, particle sizes can be classified and stored into ten different registers. A rotating scale selector determines the size range of each register.

#### 3.3.1.2 System Evaluation

To determine the degree of complexity of the manual operations required for sample processing, two procedures were evaluated. In the first, the reacted sample was removed from the suspending fluid by filtration. After drying, the filter was cleared (made transparent) with refractive index oil and a microscope focussed on the sample for analysis. In the second method, the reacted sample was placed in a microchamber (prepared by using spacers to create a chamber between a microscope slide and coverslip). After allowing several minutes for settling, microscope analysis was initiated. Both methods yielded satisfactory results, providing a degree of flexibility if this process were to be mechanized in the future.

The comparison of the Millipore system with the manual microscope method of quantitating latex agglutination was conducted by parallel evaluation of identical PIA reactions. With the manual microscope method, mean clump size was determined through the standard particle size distribution analysis technique. With the Millipore system, three different parameters were used for the evaluation: average area per clump; percent clumps per total particles (where total particles is the number of clumps plus the number of singles); and percent of clumps per total particles, (based on a linear measurement distribution of the longest horizontal chord).

A summary of the evaluation data is presented in Table 6. Two antigen-antibody systems, BSA latex and the myxovirus latexes, were used in the comparison. In the BSA latex system, a sensitized bead suspension (anti-BSA) was mixed with  $10^4$  BSA molecules at 0 time, and the reaction was monitored periodically. Both the manual and automated monitoring methods showed a specific increase in agglutination after 30-40 minutes. With the myxovirus system, varying virus concentrations were used. Two different Millipore measurements were compared with the manual microscope after a 60 minute incubation. These data are shown in Figure 6. With aliquots of identical samples, both Millipore systems indicated comparable sensitivities to the microscope method and yielded parallel responses throughout the entire range of antigen concentrations. Although a lengthy evaluation was not performed, it was felt that the Millipore system could function adequately as a sensor for the PIA reaction.

### 3.3.2 ROYCO and HIAC Particle Sensors

Two commercially available particle sensors (Royco and HiAc) were evaluated to test for feasibility as a readout for the PIA test. The two sensors were evaluated with respect to ease of operation, system sensitivity and cost of operation. The results of the tests showed that the Royco system was easier to operate because of its more flexible dilution and sampling system. The potential system sensitivity of the HiAc was greater than that of the Royco because of the higher count efficiency (95%) of the HiAc vs. 15% of the Royco. The cost of operation for the two systems

TABLE 6  
COMPARISON OF THE MILLIPORE VS THE MANUAL MICROSCOPE PROCEDURES FOR  
QUANTITATING THE PIA REACTION, USING THE MYXOVIRUS AND BSA LATEX SYSTEMS

| Time,<br>Min | BSA Latex System <sup>1</sup>              |  | Myxovirus Latex System <sup>2</sup>      |  |  |  |
|--------------|--|--|--|--|--|--|
|              | Mean Clump<br>Size (Signal<br>Minus Noise) | % Clumps/ <sup>3</sup><br>Total Particles<br>(Millipore) | Concentration<br>(EID <sub>50</sub> /ml) | Mean Clump<br>Size (Signal<br>Minus Noise) | Average Area/ <sup>4</sup><br>Clump<br>(Millipore) | % Clumps/ <sup>5</sup><br>Total Particles<br>(Millipore) |
| 0            | -  | 29   | Control                                  | -  | 7.5  | 47.0   |
| 5            | -  | 40   | 10 <sup>7</sup>                          | 0.08                                       | 7.6  | 42.0   |
| 10           | 0.09                                       | -  | 10 <sup>5</sup>                          | -  | 9.4  | 63.6   |
| 15           | -  | 28   | 10 <sup>3</sup>                          | 1.13                                       | 12.6   | 83.2   |
| 20           | 0.07                                       | -  | 10 <sup>1</sup>                          | 0.32                                       | 12.4   | 83.0   |
| 25           | -  | 28   | 10 <sup>0</sup>                          | -  | 8.9  | 47.7   |
| 30           | 0.12                                       | 46   | 10 <sup>-1</sup>                         | 0.04                                       | 8.6  | 45.0   |
| 40           | 0.42                                       | 42   | 10 <sup>-2</sup>                         | -  | 8.4  | 46.9   |
| 50           | 0.55                                       | 70   | 10 <sup>-3</sup>                         | 0.03                                       | -  | -  |
| 60           | -  | 69   | -  | -  | -  | -  |

<sup>1</sup> Anti-BSA gamma globulin, covalently bonded to 1.1  $\mu$  (diameter) polystyrene latex; 10<sup>4</sup> molecules of BSA as antigen; reaction temperature, 25C.

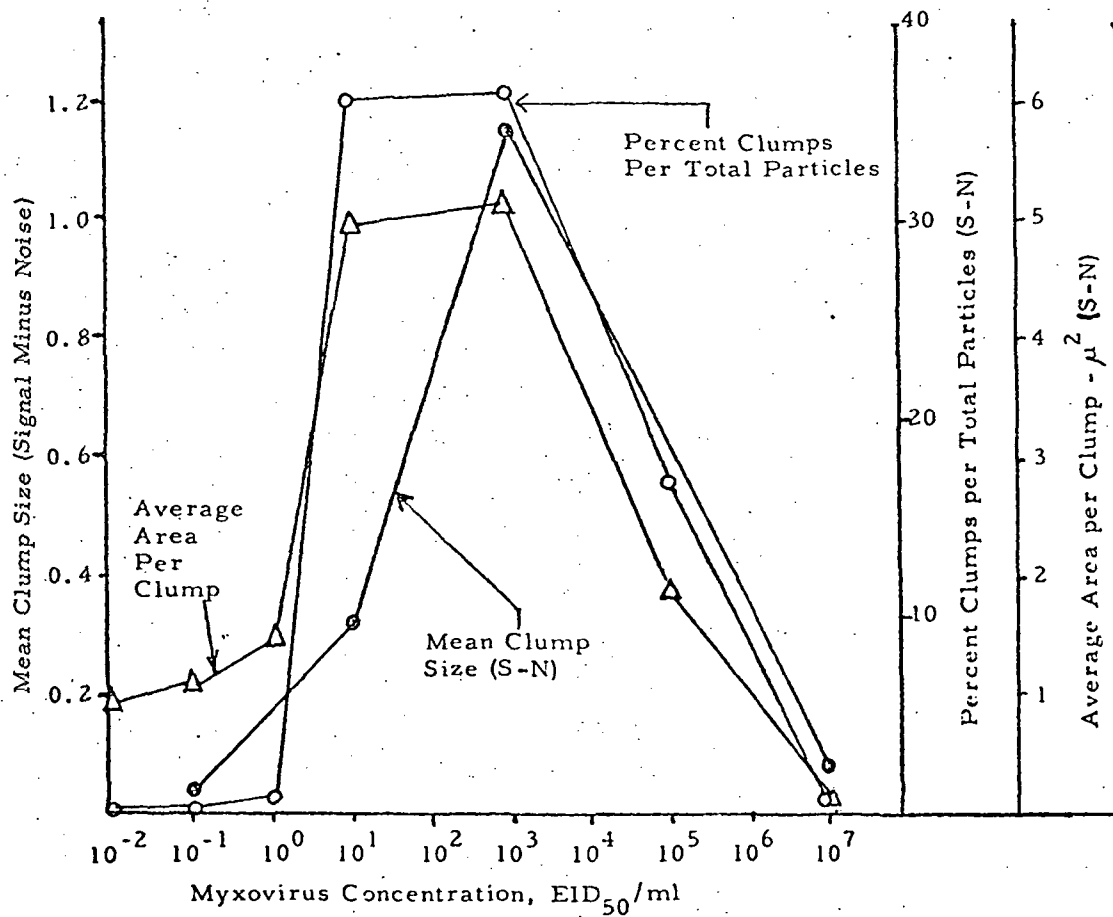
<sup>2</sup> Anti-myxovirus gamma globulin (pool 10), adsorbed to 2.02  $\mu$  (diameter) polyvinyl toluene beads; reaction temperature, 25C; 60 minutes reaction.

<sup>3</sup> Based on a particle size (area) distribution measurement; expressed as the percent clumps per total particles, where a particle represents a single bead or single clump.

<sup>4</sup> Direct instrument reading.

<sup>5</sup> Based on a particle size (horizontal chord) distribution measurement; expressed as the percent clumps per total particles, where a particle represents a single bead or single clump.

FIGURE 6  
COMPARISON OF PIA REACTION QUANTITATION MODES



Legend:

Manual Microscope Procedure (mean clump size S-N) ●—●

Π MC System, Percent Clumps per Total Number of Singles and clumps, S-N ○—○

Π MC System, Average Area per Clump (microns<sup>2</sup>), S-N △—△

was approximately the same. The initial costs of the machines were also equivalent.

The overall system performance of both counters was sub-optimal. There were two reasons for this. One was the break up of clumps during the sensing operation of the counter. The other, and more serious, problem was the inability of the present electronics to handle the large slow pulses that were produced by the clumped beads.

### 3.3.2.1 System Description

The manual method of counting bead clump sizes using a microscope is limited by time, and it is difficult to count clump sizes with more than 8 subunits. Therefore, an approximation method was used to calculate the statistic, mean clump size (MCS). In this approximation, clump sizes ranging from monomers to octamers were accurately listed and labeled, whereas larger clump sizes were given the same label and used as one number in the approximation formula to calculate the MCS.

It was reasoned that particle counters would give a measure of the total distribution of clump sizes, and thus increase precision and obviate the need for approximation formulas to calculate the statistics that were necessary to monitor the PIA reaction.

The true mean clump size is equal to the total number of beads (independent of their clumped or unclumped states) divided by the total number of particles (the term particle includes single beads and all clump sizes). The measure of clumping is inversely proportional to the number of particles because the total numbers of beads or subunits in a reaction do not change.

The analog signal which leaves the mainframe (sensor) of a particle counter is proportional to the light blocked by the particle as it passes through the sensor. Thus, the summation of the millivolts of all pulses representing particles can be used in the ratio,

$$\frac{\text{Total Millivolts}}{\text{Total Particles}},$$

to monitor a PIA reaction.

By using a pulse height analyzer (PHA) to digitize, count, and categorize the analog signals leaving the sensor, PIA reactions were rapidly monitored by the visual display of counts versus channel millivolts. As a reaction progressed, counts in low millivolt channels diminished as counts in higher millivolt channels appeared indicating that agglutination had taken place. In addition, if equal reaction aliquots representing the same number of beads were added to the sensor for each test, then the reduction in counts alone could indicate the degree of PIA reaction. The sensor, however, can read only a few thousand counts at a time without coincidence error. The bead concentration in the reaction is about  $10^9$  per ml. The difficulties associated with making a large dilution accurately prevented the use of this measurement. It was decided to sample the reaction mixture with a platinum (wire), normalize the counts, and use the statistic (average millivolts/particle) instead of absolute measurements.

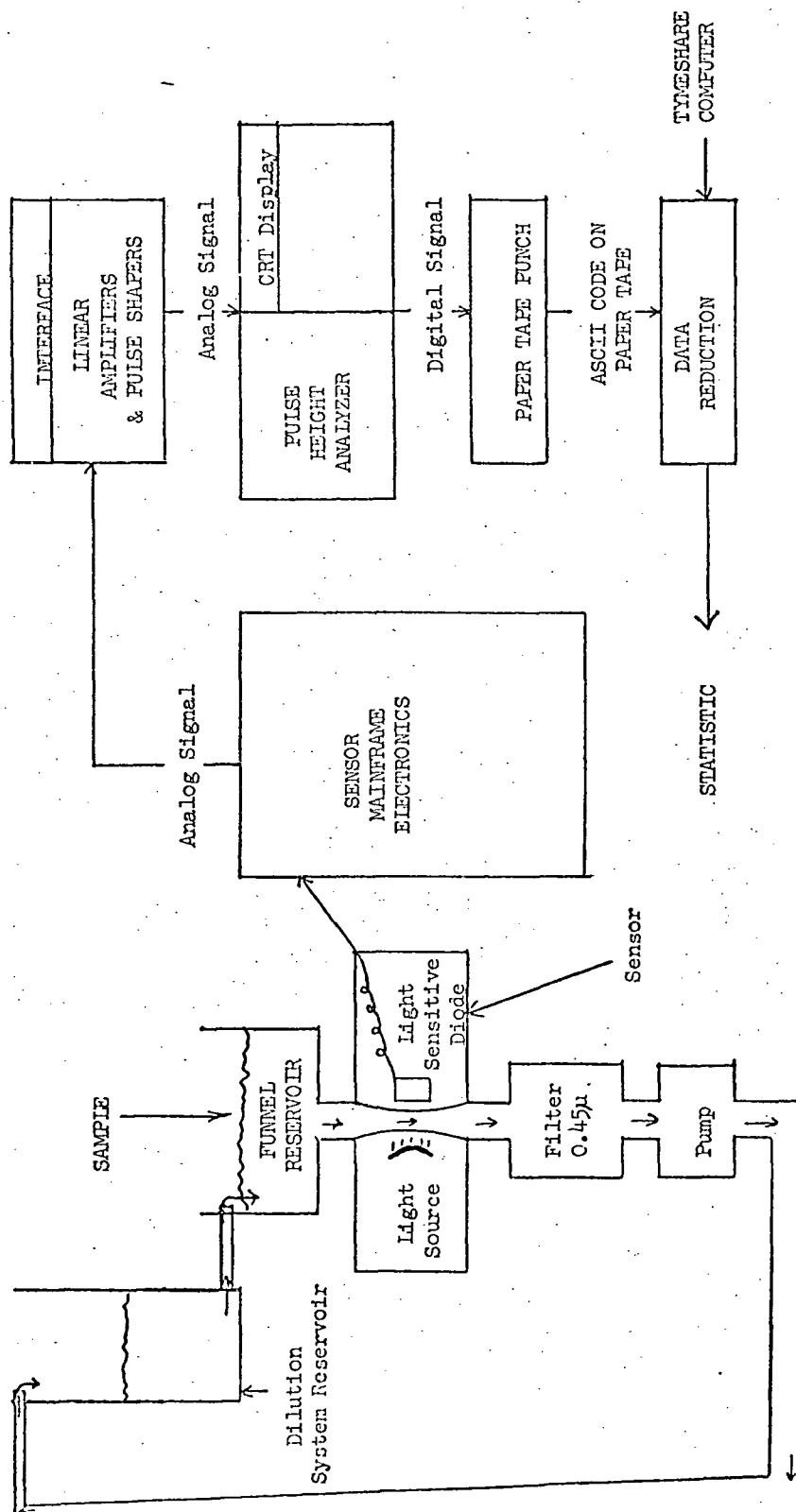
The funnel reservoir was maintained with particle free buffer. The dilution system filtered the buffer after it passed through the sensor counting window. The flow rate through the Royco sensor was 100ml/min, and within 2 minutes after adding a sample, a new sample could be introduced. The HiAc sensor required a slower, 10-15 ml/min, flow rate and longer periods were required to clear the sensor.

Figure 7 is a schematic illustrating the operation of dynamic particle counting by light blockage.

### 3.3.2.2 System Evaluation

The manual support operations are equivalent to previously described PIA reaction protocols. In this way there was a direct comparison with the manual PIA. Either before or after the manual test, the sampling platinum wire was touched to the reaction mixture then inserted into the funnel reservoir above the sensor. The results were observed in the CRT (Cathode Ray Tube), then the memory of the PHA (which contained counts corresponding to the different particle sizes) was punched onto paper tape. The TYMESHARE computer was used to sum up all the counts in the channels corresponding to different millivolt levels. Individual channel

FIGURE 7  
OPERATIONAL DIAGRAM OF ROYCO AND HIAC  
PARTICLE COUNTERS



counts were divided by the sum of all counts to normalize. The channels were assigned values corresponding to the pulse outputs of the particle sensor, and were multiplied times the counts of the corresponding channel to weight the distribution. The summation of the normalized counts multiplied by the corresponding millivolts equals the statistic:

$$\frac{\text{Total Millivolts}}{\text{Total Particles}} \text{ or } \frac{\text{Average Millivolt}}{\text{Particle}}$$

During early feasibility studies counts were weighted by channel instead of millivolts. However, the results are comparable within each experiment.

For calibrating the systems, beads were clumped immunologically to determine the channel location of the different sized clumps.

The following chart (Table 7) was made with the assumption that the particle passes through the sensor oriented such that light blockage is at a maximum.

TABLE 7  
LOCATION OF DIFFERENT  
PARTICLE SIZES BY CHANNEL NO.

| No. of Beads<br>in a Particle | Channel No. |
|-------------------------------|-------------|
| 1                             | 5-6         |
| 2                             | 7-8         |
| 3                             | 13-14       |
| 4                             | 15-16       |
| 5                             | 17-18       |
| 6                             | 19          |
| 7                             | 20          |
| 8                             | 21          |
| 9                             | 22-23       |
| 10                            | 23-24       |



It is important to realize that antibodies were used to agglutinate the antigen coated beads during the calibration experiments. The same success was not forthcoming when the antigen detection systems using antibody coated beads were compared directly with the manual PIA in the succeeding experiment.

During evaluation of the Royco system, certain problems arose, both biological and mechanical in nature. High counts appeared in channels representing low millivolt levels. The counts were sufficiently high to dwarf results in other channels when the counts were normalized. The appearance of these low millivolt counts was random. This source of error was due to a buildup of gas below the sensor window. Evidently the gas pool releases small bubbles about 1  $\mu$  in size, that rise back through the sensor window producing high numbers of counts at low signal levels. The gas pool could be removed by tilting the sensor or by momentarily increasing the flow. Table 8 illustrates the problem as different numbers of 2.02  $\mu$  beads were counted (for 2 min.) in serial tests. The average number of millivolts should have been the same in all tests.

TABLE 8

SERIAL DETERMINATION OF AVERAGE MILLIVOLT/PARTICLE  
IN EQUIVALENT SAMPLES USING THE ROYCO SYSTEM

| Test No. | Average Millivolt Particle | Particles Counted |
|----------|----------------------------|-------------------|
| 1.       | 34.52                      | 17,480            |
| 2.       | 36.53                      | 13,744            |
| 3.       | 46.16                      | 684               |
| 4.       | 34.25                      | 524               |
| 5.       | 46.73                      | 1,940             |
| 6.       | 21.20                      | 2,234             |
| 7.       | 42.98                      | 1,147             |
| 8.       | 46.86                      | 5,212             |
| 9.       | 44.37                      | 1,435             |

On the basis of this experiment, it was realized that additional engineering would be needed to make the instrument useful on a routine basis.

Another problem with Royco counting was the fact that bead clumps were breaking up upon dilution. The Royco dilution system was equilibrated with 5% Ferric Chloride to fix the particles in their clumped state. Table 9 illustrates marginal detection of  $10^2$  CFU of Mycoplasma using Ferric Chloride as a diluent of the manual PIA reaction mixture.

TABLE 9  
COMPARISON OF THE ROYCO PARTICLE SENSOR  
AND THE SAMPLE  $-10^2$  CFU OF M. PNEUMONIAE

| Sample             | Royco<br>Av. Mv/particle | Manual Mean<br>Clump Size |
|--------------------|--------------------------|---------------------------|
| Signal             | 45.8                     | 2.73                      |
| Noise              | 44.8                     | 2.46                      |
| Signal minus Noise | 1.0                      | 0.27                      |

Great care was made to remove all bubbles from the sensor and the profiles indicated a slightly higher signal when M. pneumoniae ( $10^2$  CFU) antigen was in the reaction in place of the control.

The M. pneumoniae latex system ( $10^2$  CFU) was also used with the HiAc particle counting system. However, instead of using ferric chloride to fix the clumping, the PIA reaction was allowed to proceed for 48 hours. The control sample equalled 14.6 mv/particle while the sample reached 15.8 mv/particle. The HiAc counted 95-100% of the particles passing through the aperture compared with 15% on the Royco. These experiments concur with the scientific literature which indicates that the antigen-antibody reaction increases stability with time. The results with both systems, however, remained marginal.

The work with the Royco and HiAc counters indicated that a dynamic system of this type offered considerable problems with both the engineering and the biology. In choosing between the Royco and HiAc particle counters, the HiAc showed the greatest promise.

### 3.3.3 ELECTROZONE CELLOSCOPE

#### 3.3.3.1 System Description

The Celloscope System diagramed in Figure 8, as used for quantitating the PIA reaction, is comprised of the Celloscope transducer/sensor (Particle Data), oscilloscope (Hewlett Packard), and a signal processor and display system (Nuclear Data).

The Celloscope transducer Model III has a cylindrical aperture separating two electrode chambers filled with electrolyte (Figure 9). The particles being measured are suspended in the outer chamber and then drawn through the aperture, through which an electric current also passes. As each particle goes through the orifice, it changes the volume (and hence resistance) of the small hemispherical volume at either end of the orifice. An electrical pulse is generated with an amplitude proportional to particle volume. The sequence of particle pulses is then amplified, scaled and counted or otherwise processed electronically to yield particle count/size data. The equation for the amplitude of the amplifier output signal voltage is:

$$e = \frac{IGvl_c}{A^2} \left( \frac{1}{1-l_c/l} - \frac{a}{A} \right)^{-1}$$

I = Electrozone current

G = Amplifier gain

v = Particle volume

$l_c$  = Electrolyte resistivity (compensated via circuitry)

A = Orifice area

l = Particle resistivity

a = Particle area

The Nuclear Data Series 4410 system is a single parameter data acquisition and display system. The basic system consists of a 50 MHz<sub>z</sub> analog to digital converter, and ND-812 4K central processor, a control module, a bin mounted power supply and a Hewlett Packard display oscilloscope.

FIGURE 8  
SCHEMATIC OF THE CELLOSCOPE SYSTEM

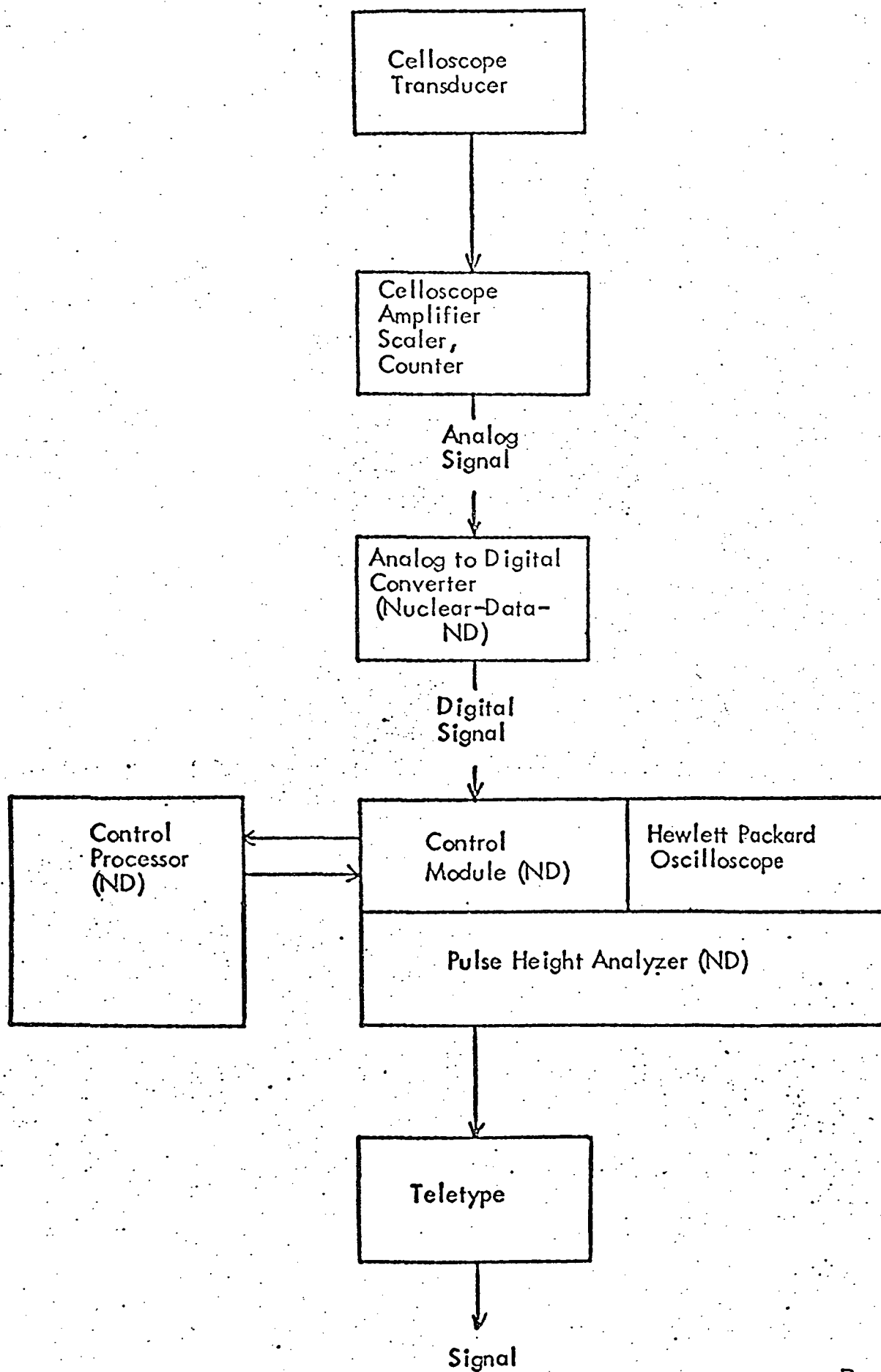
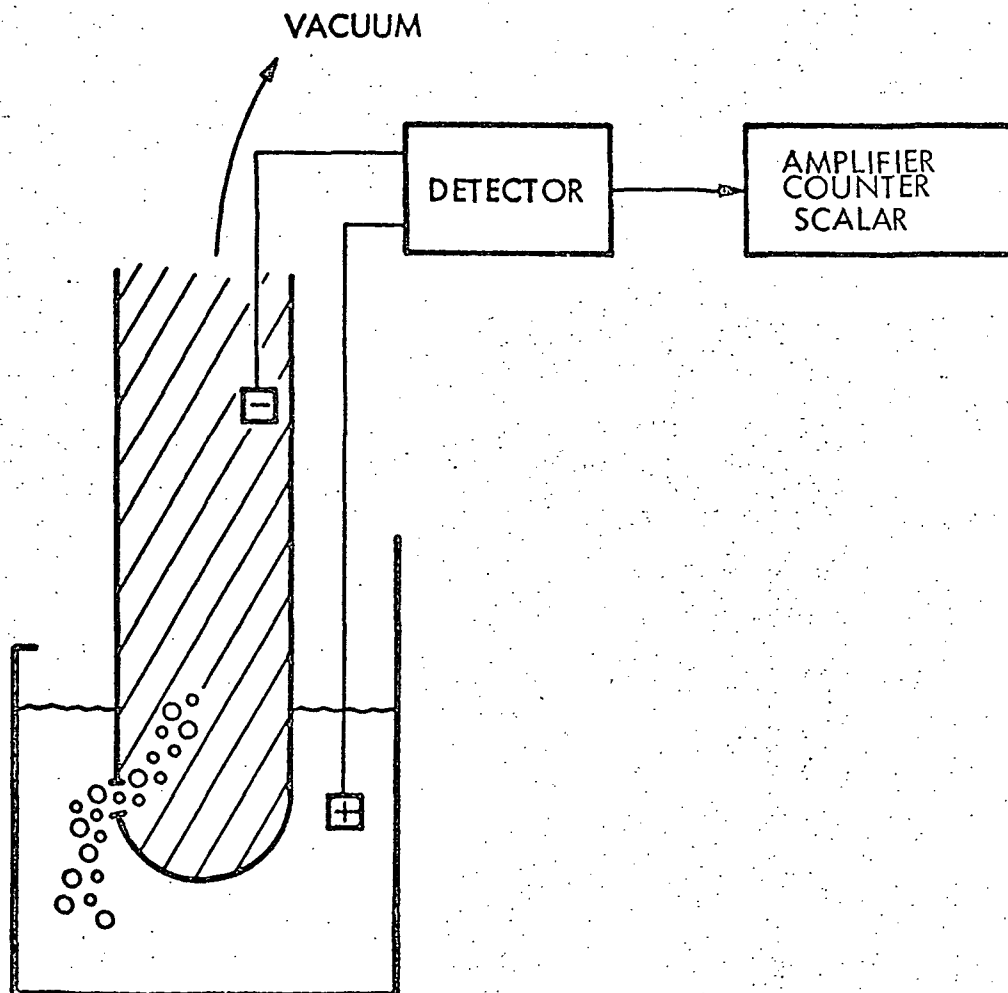


FIGURE 9  
SCHEMATIC OF THE CELLOSCOPE  
MEASUREMENT PRINCIPLE



The analog to digital converter module (ADC) is designed primarily for the processing of amplitude modulated signal pulses, such as encountered in pulse height analysis. It contains an analog to digital converter and a 12 bit address scaler.

The basic ND-812 central processor contains a 12-bit, 4K memory. Core locations are directly addressable by using two-word instructions. A total of 256 single word or 4096 two-word I/O commands are possible. Other features include the 12 or 24-bit programmed I/O transfer, a four level priority interrupt, four micro-programmable pulses per I/O instruction, direct memory access, four arithmetic registers hardware multiply and divide and 100% integrated control logic circuitry.

The control module interfaces the ADC's and display scope with the ND-812 central processor. The module establishes the number of channels displayed, increases or decreases the number of counts fill scale and positions two data makers. The data contained between the markers can be totalized, read out by channel or altered by addition, subtraction, division and multiplication.

### 3.3.3.2 System Evaluation

A direct comparison of the Celloscope with the manual PIA was performed. Both tests employed the same manual support operations. From a given sample (after mixing and incubation) an aliquot (0.025 ml) was removed for microscopic analysis. For the Celloscope, an identical volume from the same sample was diluted to  $10^4 - 10^6$  particles/ml in diluent buffer (particle free) and processed.

The processing is accomplished by placing the Celloscope transducer in the diluted sample. A 30 second count is made at a given flow rate ( 0.1-2 ml/min). The frequency distribution of the 256 channel pulse height analyzer is displayed on the oscilloscope. From the distribution, the data markers (described in control module) were set for the specific channel locations at the boundaries of the first peak. The first peak corresponds to the frequency of singles or non-clumped beads. The boundaries of the first peak are set once only before a series of experiments.

To characterize a given distribution, the following four parameters were selected for deriving a single (yet representative) statistic.

$$\begin{aligned}
 \text{a. Total Count} &= \sum_{i=1}^{256} Y_i \\
 \text{b. Normalized Singles} &= \sum_{i=1}^n \frac{Y_i X_i^4}{\sum_{i=1}^{256} Y_i} \\
 \text{c. Normalized Clumps} &= \sum_{i=n+1}^{256} \frac{Y_i X_i^4}{\sum_{i=1}^{256} Y_i} \\
 \text{d. Ratio} &= \frac{\sum_{i=n+1}^{256} Y_i X_i^4}{\sum_{i=1}^n Y_i X_i^4}
 \end{aligned}$$

where  $X_i$  = channel number  
 $Y_i$  = frequency  
 $n$  = channel number of the right hand data marker

In an agglutinated sample, where the number of clumps is increasing, there is a corresponding increase in the ratio. The ratio is used since the exact number of beads per specimen is extremely difficult to control, rendering a statistic such as a singles decrease useless. However, the ratio enables the comparison of all data counted under similar experimental conditions regardless of bead concentration.

The initial feasibility studies were conducted with salt agglutinated beads. Polyvinyltoluene latex particles ( $2.02 \mu$  in diameter) were sensitized with anti-mycoplasmal antibody. These beads were suspended in three concentrations of NaCl (0.1%, 1%, and 5%). Instrument conditions, reaction times, etc. were varied among the different experiments, so an absolute correlation from run to run was not anticipated. The absolute ratios or mean clump sizes were normalized for comparative purposes only. The summary data for the salt series are presented in Table 10. The control consisted of diluting the beads in distilled water. From these data, it is apparent that the higher salt concentrations cause an increase in mean clump size (PIA microscope) and Celloscope ratio. In Figure 10, these data are graphically depicted. The linear regression was performed on the signal corresponding to the three salt concentrations. The correlation coefficients of 0.97 (Celloscope) and 0.94 (PIA microscope) indicate a linear response of increasing salt concentration with the Celloscope ratio or standard mean clump size. The slopes and intercepts between the two quantitation modes are also similar, with the indication that the Celloscope ratio at least monitors changes caused by salt concentration to the same degree as the PIA microscope.

The comparison of the Celloscope with the microscope PIA for quantitating immunologically clumped beads was performed utilizing the myxovirus system. It should be emphasized that comparison between individual runs cannot be made since the conditions between the runs were radically varied. However, the comparison of the overall change in signal by increasing virus concentration should be similar between the



TABLE 10  
COMPARISON OF CELLOSCOPE AND MANUAL PIA QUANTITATION OF SALT CLUMPED BEADS

| Run No. | Celloscope - Raw Data (Expressed as Ratio) |                    |                    |                    |
|---------|--|--------------------|--------------------|--------------------|
|         | Control                                    | Concentration NaCl |                    |                    |
|         |  | 0.1%               | 1.0%               | 5.0%               |
| 1       | $2.38 \times 10^2$                         | $2.78 \times 10^2$ | $8.92 \times 10^2$ | $2.13 \times 10^3$ |
| 2       | $7.38 \times 10^2$                         | $8.82 \times 10^2$ | $2.45 \times 10^2$ | $5.05 \times 10^3$ |
| 3       | $6.81 \times 10^2$                         | $7.84 \times 10^2$ | $2.25 \times 10^3$ | $3.75 \times 10^3$ |

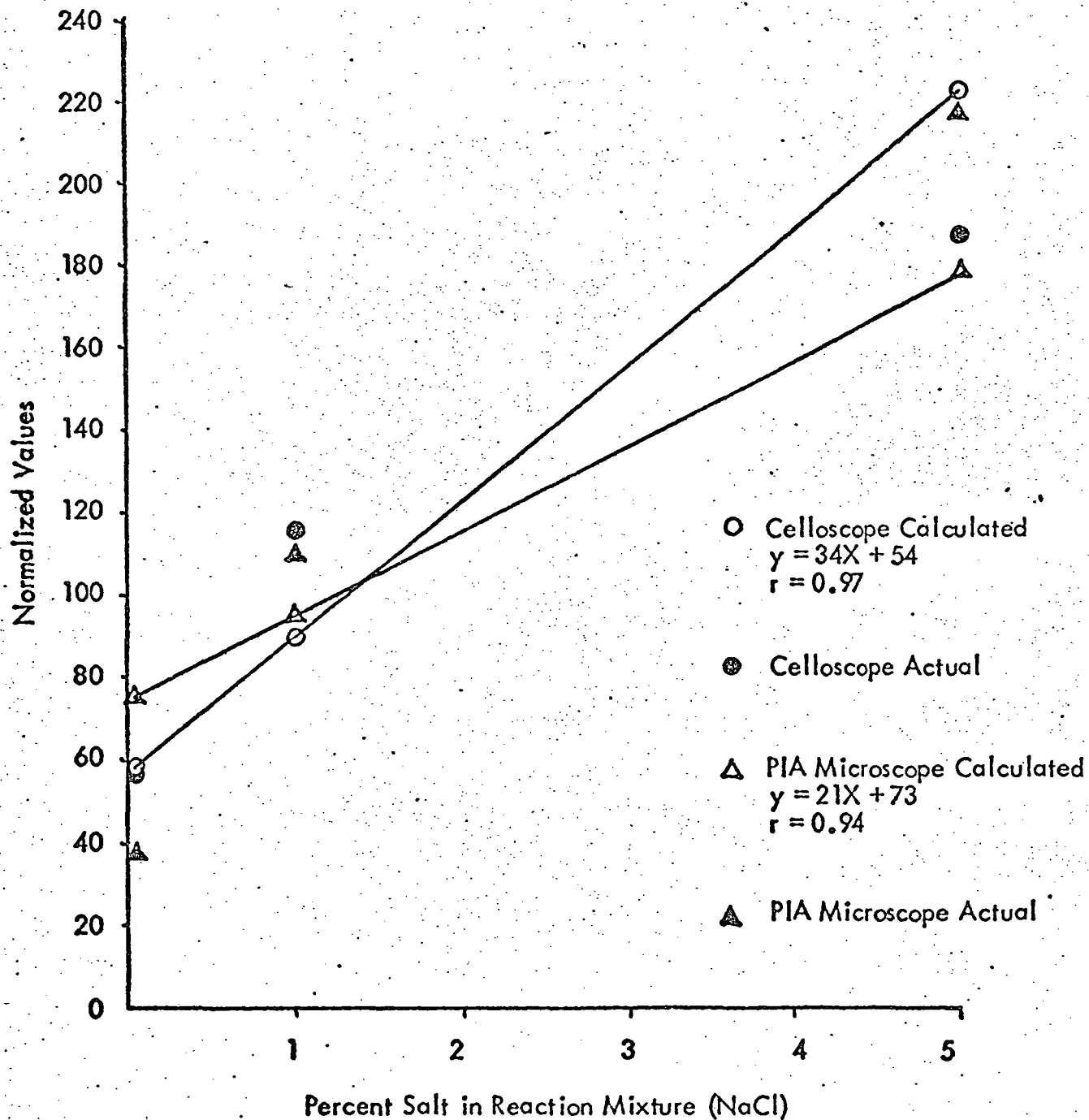
| Celloscope - Normalized Data |         |      |      |      |
|------------------------------|---------|------|------|------|
| Run No.                      | Control | 0.1% | 1.0% | 5.0% |
| 1                            | 14      | 16   | 50   | 127  |
| 2                            | 44      | 53   | 146  | 302  |
| 3                            | 41      | 47   | 135  | 224  |
| Column Means                 | 33      | 38.7 | 110  | 218  |

| PIA Microscope - Raw Data (Expressed as Mean Clump Size) |                    |      |      |      |
|--|--------------------|------|------|------|
| Run No.  | Concentration NaCl |      |      |      |
|  | Control            | 0.1% | 1.0% | 5.0% |
| 1  | 1.61               | 2.12 | 3.58 | 9.72 |
| 2  | 1.66               | 2.05 | 3.69 | 3.77 |
| 3  | 1.77               | 2.02 | 4.74 | 4.98 |

| PIA Microscope - Normalized Data |         |      |      |      |
|----------------------------------|---------|------|------|------|
| Run No.                          | Control | 0.1% | 1.0% | 5.0% |
| 1                                | 46      | 61   | 103  | 280  |
| 2                                | 48      | 59   | 106  | 108  |
| 3                                | 51      | 58   | 136  | 143  |
| Column Mean                      | 48      | 59   | 115  | 177  |

FIGURE 10

COMPARISON OF CELLOSCOPE AND PIA MICROSCOPE  
AS QUANTITATION MODES FOR SALT CLUMPED BEADS  
USING NORMALIZED VALUES



PIA microscope and Celloscope. In Table 11 and Figure 11, the results for the myxovirus comparison are presented. The slopes and Y intercepts are nearly identical for both quantitation modes. These data indicated that the Celloscope System was suitable for quantitating the PIA reaction. It should be noted that test reliability should favor the Celloscope since  $10^5 - 10^6$  beads are sampled compared to the  $10^2$  for the microscope PIA. The correlation coefficients were high, indicating a linear relationship between virus concentration and both the mean clump or Celloscope ratio.

### 3.3.4 IMPROVED CELLOSCOPE SYSTEM

#### 3.3.4.1 System Description

The Nuclear Data Central Processing Unit was reprogrammed so that a given frequency distribution could be characterized with greater flexibility. Four parameters are still used for deriving the final statistic in which the first (total count) and the fourth (the ratio of the second and third parameters) are the same as previously described. The second parameter measures the percent of singles between the left and right markers. The third parameter measures the normalized clumps from the right hand marker to channel 256. The flexibility of the new program is demonstrated by the ability to increase or decrease the exponent of  $X_i$ , for arriving at the optimum signal to noise (S/N) ratio with a simple operation. A series of preliminary tests was conducted where  $X_i^6$  ( $X_i$  = channel number) provided the highest S/N ratio. The use of the  $10^6$  weighting factor is now characterized by near optimum signal and noise values.

#### 3.3.4.2 System Evaluation

A series of tests were performed to determine the specificity of the latex test for the myxovirus system as quantitated by the Celloscope.

TABLE 11  
COMPARISON OF THE CELLOSCOPE AND MANUAL PIA  
QUANTITATION OF THE MYXOVIRUS LATEX SYSTEM

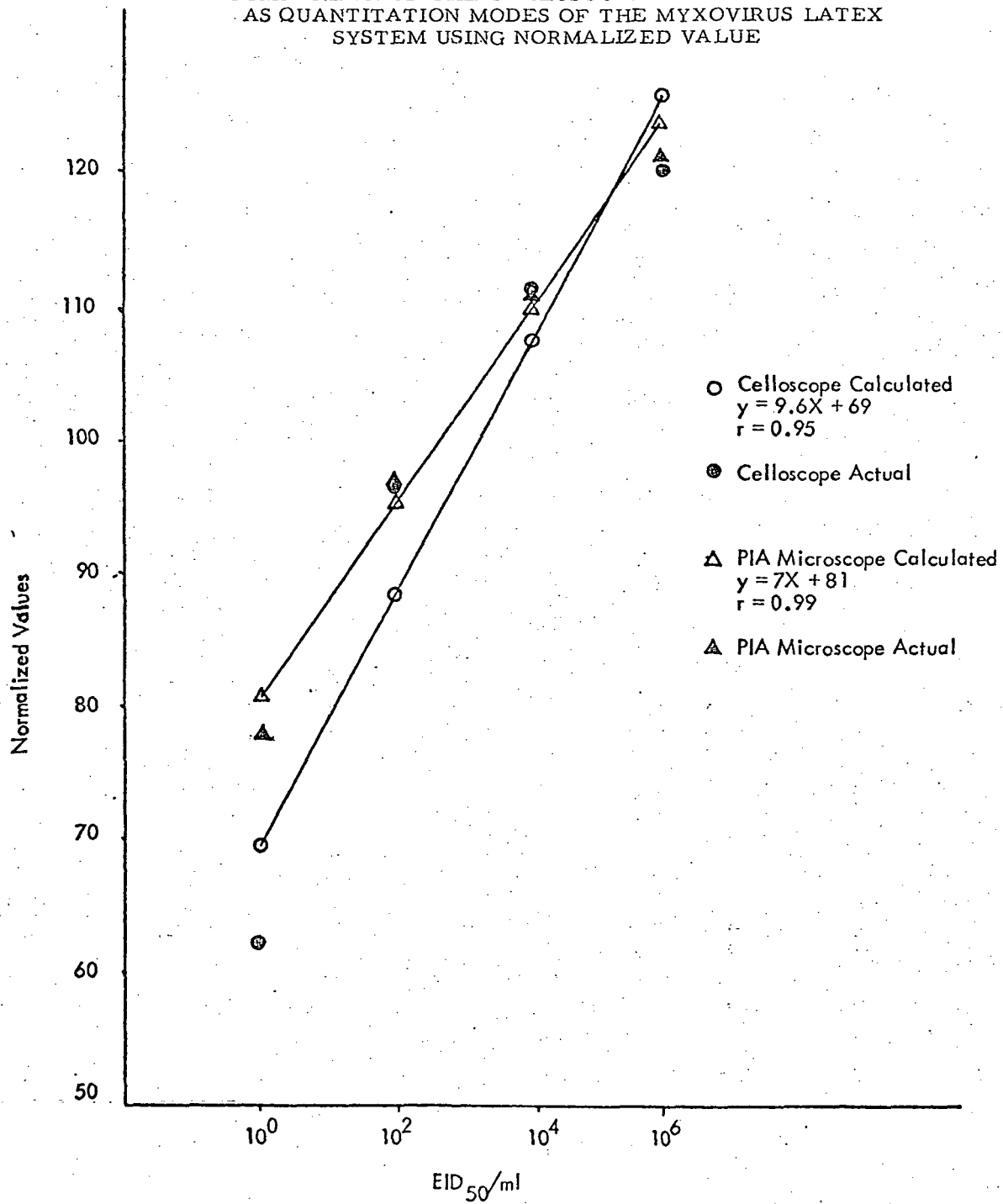
| Run No.     | Celloscope - Ratio                   |       |                                      |      |                                      |      |                                      |      |
|-------------|--------------------------------------|-------|--------------------------------------|------|--------------------------------------|------|--------------------------------------|------|
|             | Concentration                        |       |                                      |      |                                      |      |                                      |      |
|             | 10 <sup>0</sup> EID <sub>50/ml</sub> |       | 10 <sup>2</sup> EID <sub>50/ml</sub> |      | 10 <sup>4</sup> EID <sub>50/ml</sub> |      | 10 <sup>6</sup> EID <sub>50/ml</sub> |      |
|             | Raw                                  | Norm* | Raw                                  | Norm | Raw                                  | Norm | Raw                                  | Norm |
| 1           | 53                                   | 0.9   | 38                                   | 0.7  | 46                                   | 0.8  | 97                                   | 1.7  |
| 2           | 40                                   | 0.7   | 47                                   | 0.8  | 42                                   | 0.7  | 300                                  | 5.2  |
| 3           | 84                                   | 1.5   | 90                                   | 1.6  | 81                                   | 1.4  | 613                                  | 11   |
| 4           | 25,800                               | 467   | 27,800                               | 483  | 34,500                               | 599  | 21,600                               | 375  |
| 5           | 15,000                               | 260   | 15,200                               | 264  | 14,800                               | 257  | 14,100                               | 245  |
| 6           | 1,700                                | 30    | 1,870                                | 32   | 2,160                                | 37   | 4,400                                | 76   |
| 7           | 1,320                                | 23    | 696                                  | 12   | 1,150                                | 20   | 15,400                               | 267  |
| 8           | 1,820                                | 32    | 1,920                                | 33   | 2,400                                | 42   | 1,190                                | 21   |
| 9           | 2,600                                | 45    | 2,430                                | 42   | 2,740                                | 48   | 5,210                                | 90   |
| Column Mean |                                      | 62    |                                      | 97   |                                      | 112  |                                      | 121  |

| Run No.     | PIA - Microscope (Mean Clump Size)  |      |                                      |      |                                      |      |                                      |      |
|-------------|-------------------------------------|------|--------------------------------------|------|--------------------------------------|------|--------------------------------------|------|
|             | Concentration                       |      |                                      |      |                                      |      |                                      |      |
|             | 10 <sup>0</sup> ID <sub>50/ml</sub> |      | 10 <sup>2</sup> EID <sub>50/ml</sub> |      | 10 <sup>4</sup> EID <sub>50/ml</sub> |      | 10 <sup>6</sup> EID <sub>50/ml</sub> |      |
|             | Raw                                 | Norm | Raw                                  | Norm | Raw                                  | Norm | Raw                                  | Norm |
| 1           | 1.18                                | 45   | 1.22                                 | 47   | 1.26                                 | 48   | 1.20                                 | 46   |
| 2           | 1.19                                | 46   | 1.11                                 | 43   | 1.31                                 | 50   | 1.75                                 | 67   |
| 3           | 1.38                                | 53   | 1.44                                 | 55   | 1.22                                 | 47   | 1.84                                 | 71   |
| 4           | 3.97                                | 152  | 4.27                                 | 164  | 4.74                                 | 182  | 5.01                                 | 192  |
| 5           | 3.32                                | 127  | 3.76                                 | 144  | 4.73                                 | 181  | 3.76                                 | 144  |
| 6           | 2.46                                | 94   | 3.11                                 | 119  | 2.94                                 | 113  | 4.38                                 | 168  |
| 7           | 2.15                                | 82   | 2.41                                 | 92   | 3.81                                 | 146  | 4.00                                 | 153  |
| 8           | 2.37                                | 91   | 2.61                                 | 100  | 2.95                                 | 113  | 3.09                                 | 118  |
| 9           | 3.07                                | 118  | 2.90                                 | 111  | 3.18                                 | 122  | 3.44                                 | 132  |
| Column Mean |                                     | 79   |                                      | 97   |                                      | 111  |                                      | 121  |

\*Normalized Data

FIGURE 11

COMPARISON OF THE CELLOSCOPE AND PIA MICROSCOPE  
AS QUANTITATION MODES OF THE MYXOVIRUS LATEX  
SYSTEM USING NORMALIZED VALUE



Both crude virus (suspended in chorioallantoic fluid) and purified virus (suspended in PBS, pH 8, with .02% gelatin) were reacted against anti-myxovirus and anti-mycoplasma bead preparations. All reactions were conducted for 60 minutes at 40 C. The results are presented in Table 12. Both the antigen preparations indicated reactivity with S/N values of 1.49 and 1.46 against the homologous bead preparation. Against the anti-mycoplasma bead preparation the differences from control were insignificant. The antigen concentration utilized in this test series was  $10^6$  EID<sub>50</sub>/ml.

TABLE 12  
SPECIFICITY OF THE INFLUENZA LATEX SYSTEM  
AS QUANTITATED BY THE CELLOSCOPE

| Antigen        | Anti-Influenza<br>Bead Preparation | Anti-Mycoplasma<br>Bead Preparation |
|----------------|------------------------------------|-------------------------------------|
| Crude Virus    |                                    |                                     |
| Log Signal     | 12.55                              | 12.93                               |
| Log Noise      | 12.37                              | 12.92                               |
| S/N            | 1.49                               | 1.02                                |
| Purified Virus |                                    |                                     |
| Log Signal     | 12.25                              | 13.09                               |
| Log Noise      | 12.08                              | 13.09                               |
| S/N            | 1.46                               | 1.0                                 |

In evaluating the utilization of the Celloscope for quantitating the latex agglutination reaction for M. penumoniae, a test series was chosen where the antigen concentrations were  $5 \times 10^6$ ,  $10^6$ ,  $5 \times 10^5$ ,  $5 \times 10^4$  and  $10^4$ . The summary results for these data are depicted in Table 13 and Figure 12. The averaged log of the signal is compared at the six antigen concentrations. The correlation coefficient of 0.93 indicated a fairly linear relationship over the entire range. A paired-sample "t" test applied to all tests indicated that the log of the signal for  $5 \times 10^6$ ,  $10^6$ ,  $5 \times 10^5$ ,  $10^5$  and  $5 \times 10^4$  were different (95% confidence level) than  $10^4$ . In addition,  $5 \times 10^6$  was significantly different than  $10^6$ .

FIGURE 12  
 QUANTITATION OF THE MYCOPLASMA LATEX  
 AGGLUTINATION REACTION BY THE CELLOSCOPE

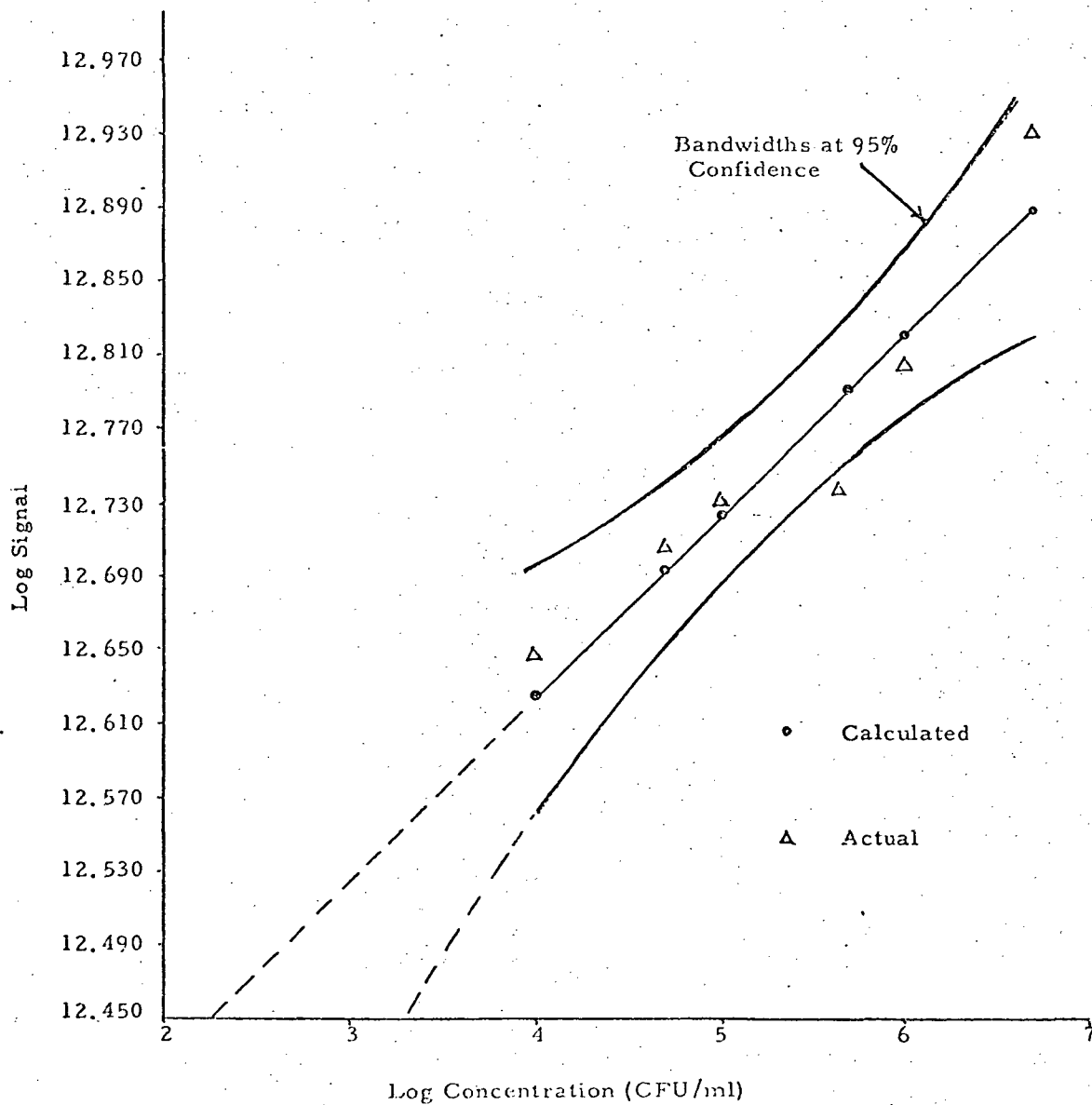


TABLE 13  
RELATIONSHIP OF CELLOSCOPE RESPONSE  
TO CONCENTRATION OF M. PNEUMONIAE

| Antigen Concentration<br>CFU/ml | Average Signal*<br>(Log)                |
|---------------------------------|---|
| $5 \times 10^6$                 | 12.937                                  |
| $1 \times 10^6$                 | 12.805                                  |
| $5 \times 10^5$                 | 12.734                                  |
| $1 \times 10^4$                 | 12.729                                  |
| $5 \times 10^4$                 | 12.701                                  |
| $1 \times 10^4$                 | 12.648                                  |
| $y = 0.97 (\log x) +$<br>12.240 | $r = 0.93$ (Correlation<br>Coefficient) |

\*Average of 11 tests

A demonstration was performed for NASA personnel using the Celloscope to quantitate the latex agglutination reaction for the Mycoplasma. The results are presented in Table 14. In the statistical analysis of the data, it was found that  $10^2$  CFU/ml were different (95% confidence level) or were distinguished from the control and  $10^0$  CFU/ml. The reproducibility of these results under identical conditions was not tested and is mentioned to prevent any possible misinterpretations. In practice, it may take a large number of tests to recognize this level or possibly a less desirable sensitivity of  $10^3 - 10^4$  may be the maximum realizable via the Celloscope quantitative mode (without additional refinement).



TABLE 14  
DEMONSTRATION OF THE CELLOSCOPE FOR  
QUANTITATING MYCOPLASMA VIA LATEX BEAD  
AGGLUTINATION

| Antigen Concentration<br>CFU/ml | Signal-to-Noise*<br>(S/N)               |
|---------------------------------|---|
| $10^6$                          | 5.98                                    |
| $10^4$                          | 3.48                                    |
| $10^2$                          | 1.80                                    |
| $10^0$                          | 1.00                                    |
| $6 = 0.82 (\log x) + 0.59$      | $r = 0.98$ (Correlation<br>Coefficient) |

\*Noise was determined by processing identical specimens without antigen.

In order to test the sensitivity of the influenza latex system in the Celloscope, 88 test series were examined. The data from these tests were also analyzed by the paired sample "t" test to facilitate overall comparisons with previous studies. Table 15 expresses the level of confidence or probability of distinguishing two narrowly dissimilar antigen concentrations. With 70% confidence 0.026 HA units of antigen were distinguishable from 0.128 HA units. The difference between 0.128 and 0.256 HA units per ml was shown with 95% confidence. The differences between 0.256 and 1.28 as well as 1.28 and 2.56 HA units/ml were observable with 99% confidence. At the highest antigen concentration (2.56 vs 12.8) the confidence level showed a slight drop to 95% giving an indication of the drop in signal corresponding to excess antigen.

TABLE 15  
PROBABILITY OF SIGNIFICANT SIGNAL DIFFERENCES OF  
VARIOUS MYXOVIRUS CONCENTRATION AS QUANTITATED  
BY THE CELLOSCOPE

| Concentration<br>(HA/ml) | "t"<br>Statistic | Degrees of<br>Freedom | % Confidence<br>or Probability<br>of Difference |
|--------------------------|------------------|-----------------------|---|
| 0.128 vs 0.026           | 1.21             | 31                    | 70%   |
| 0.256 vs 0.128           | 2.49             | 19                    | 95%   |
| 1.280 vs 0.256           | 5.02             | 24                    | 99+%  |
| 2.560 vs 1.280           | 5.68             | 36                    | 99+%  |
| 12.800 vs 2.560          | 2.17             | 35                    | 95%   |

In Table 16 percent reduction in signal is shown for decreasing antigen concentrations. This data is graphically represented in Figures 13 and 14. The log of the % reduction in signal yielded a correlation coefficient of 0.98 indicating a strong linear relationship.

TABLE 16  
CELLOSCOPE READOUT DATA

| Antigen Concentration |                 | % Signal | Log % Signal |
|-----------------------|-----------------|----------|--------------|
| HA Units/ml           | Log HA Units/ml |          |              |
| 12.800                | 1.11            | 100      | 2.000        |
| 2.560                 | 0.408           | 54       | 1.732        |
| 1.280                 | 0.107           | 16       | 1.204        |
| 0.256                 | -0.592          | 3        | 0.477        |
| 0.128                 | -0.893          | 2        | 0.301        |
| 0.026                 | -1.585          | 1        | 0.000        |

### 3.3.5 THE PHOTOMETRIC METHOD

The determination of the amount of agglutination in samples by photometric techniques involves measurements of the light transmitting

FIGURE 13  
MYXOVIRUS CELLOSCOPE DATA--  
COMPILATION OF 88 TEST SERIES

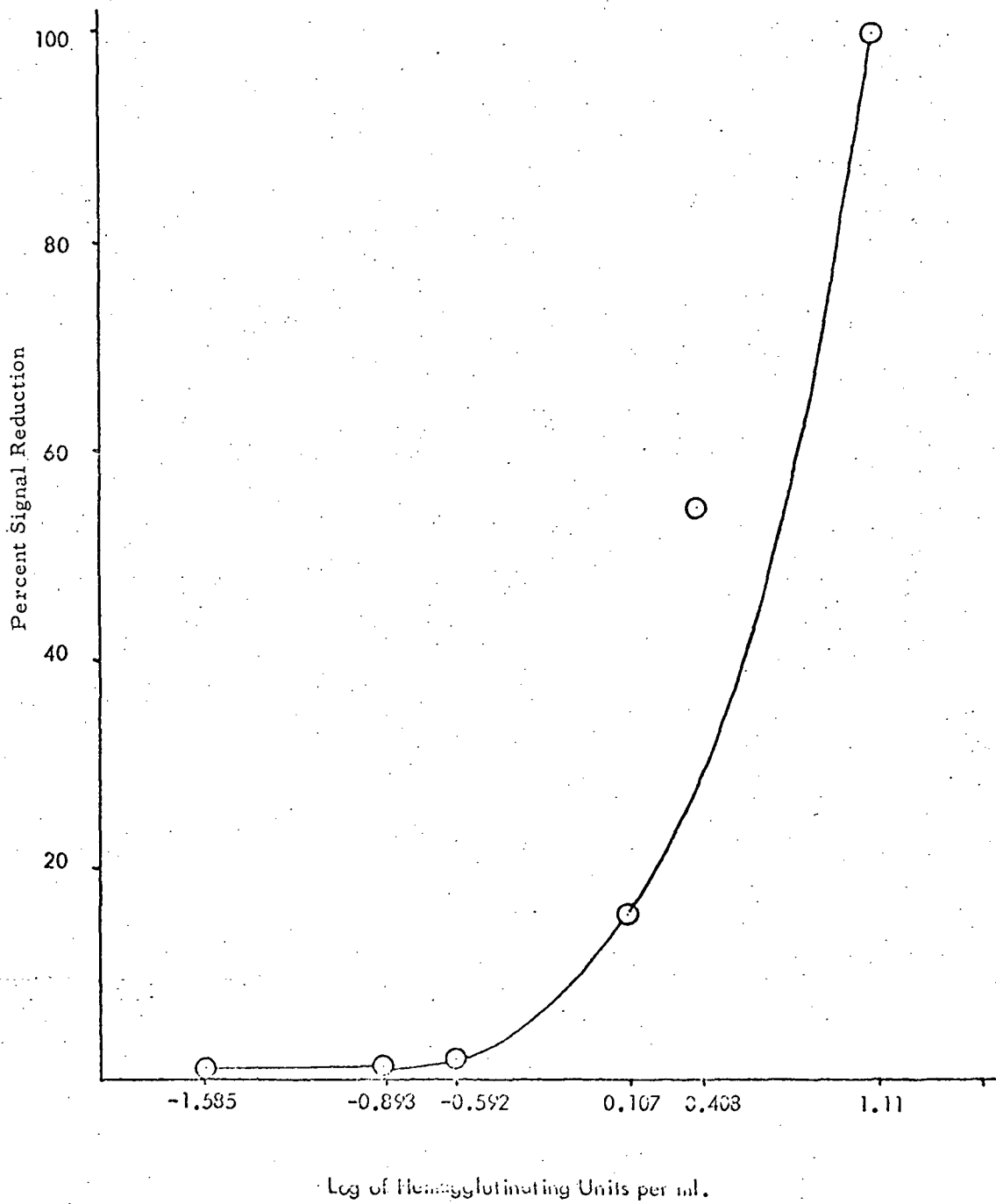
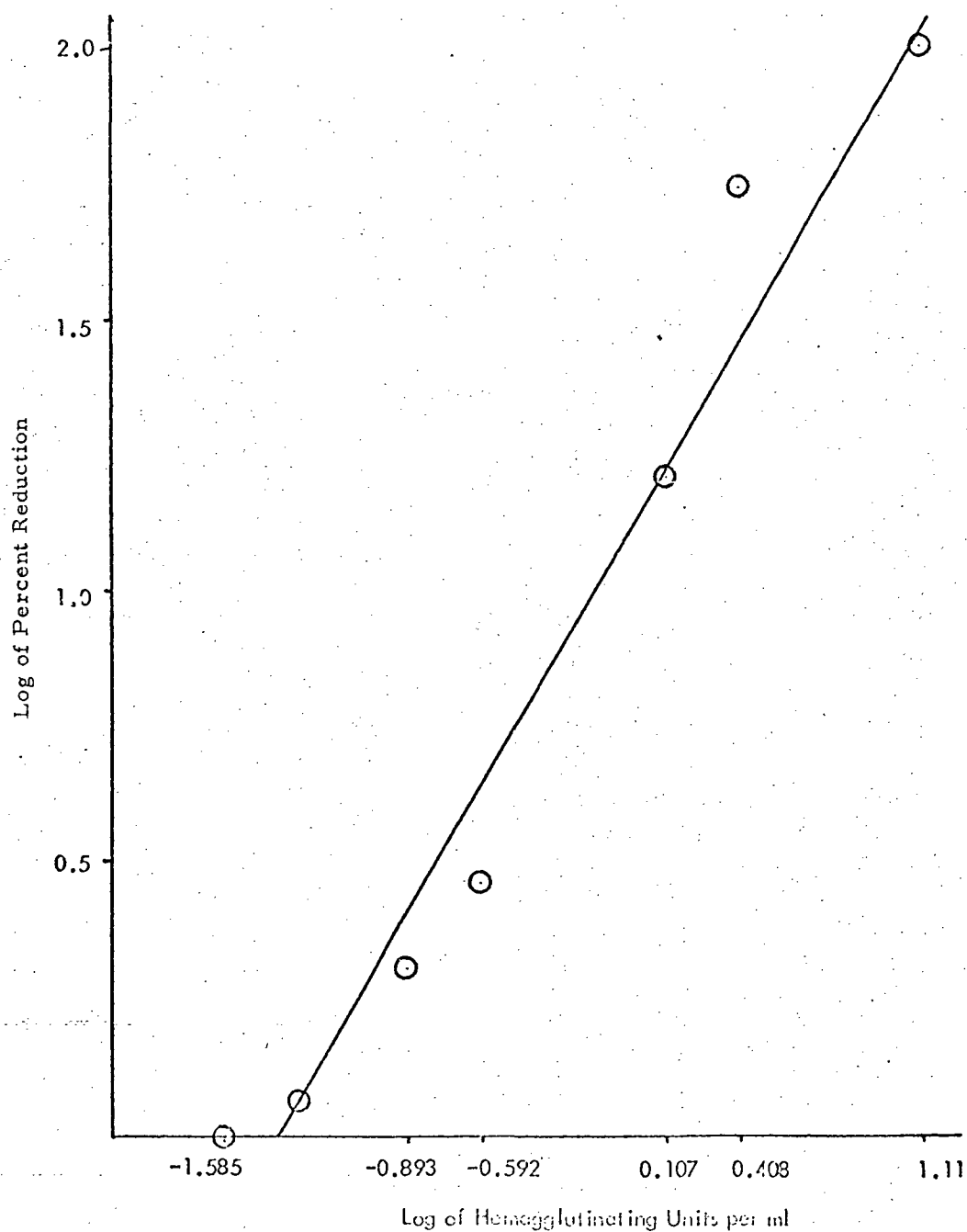


FIGURE 14  
 MYXOVIRUS CELLOSCOPE DATA--  
 COMPILATION OF 88 TEST SERIES



qualities of the sample. A comparison with standard solutions or controls can then be related to agglutination level. For studies on the photometric method the Beckman DK-2 spectrophotometer was used.

### 3.3.5.1 System Description

The photometric effects of agglutination are shown schematically in Figure 15. In the top illustration a light passes through a mono-disperse bead solution. Each bead intercepts a portion of the incident light and prevents it from reaching the detector. If these same beads are now agglutinated as in the lower illustration, the effective cross-sectional area blocking light is reduced and so the amount of transmitted light increases. Analytically this is shown in Figure 16. The optical density (O.D.) or lack of transmission in an unagglutinated bead solution is proportional to the number of beads,  $N$ , the cross-sectional area, and the extinction coefficient,  $Q$ . In an agglutinated solution two additional factors enter the optical density equations. The packing factor,  $F$ , enters because the clumping process leaves void spaces between beads. The factor  $F$  tends to raise the O.D.; however, the effect is small. The second factor is the mean clump size (beads/clump). Increased clumping or agglutination results in a larger mean clump size and reduced optical density (more light transmission).

Individual beads scatter and absorb light with scattering dominating. Mie scattering theory predicts a maximum absorption (or scattering) to occur at wavelengths comparable to the bead radius. At radius/wavelength ratios less than about 1, the scattering tends toward zero. At ratios greater than 1 the scattering is oscillatory. For particle sizes of interest ( $\sim .8 \mu$  to  $1.1 \mu$ ) the oscillations occur in the UV and exhibit erratic behavior. Slight changes in particle size or wavelength result in drastic changes in absorbance. Absorption measurements were made on suspensions of several bead sizes in the range of interest. Behavior was generally in accordance with the theory. Spectrophotometer traces of two suspensions are shown in Figures 17 and 18. Peak absorption is about as theory predicts; with  $0.8 \mu$  beads the peak occurred at about  $0.36 \mu$  ( $0.4 \mu$  theoretical) and with  $1.1 \mu$  beads, the peak occurred in the

FIGURE 15

# PHOTOMETRIC EFFECTS OF AGGLUTINATION

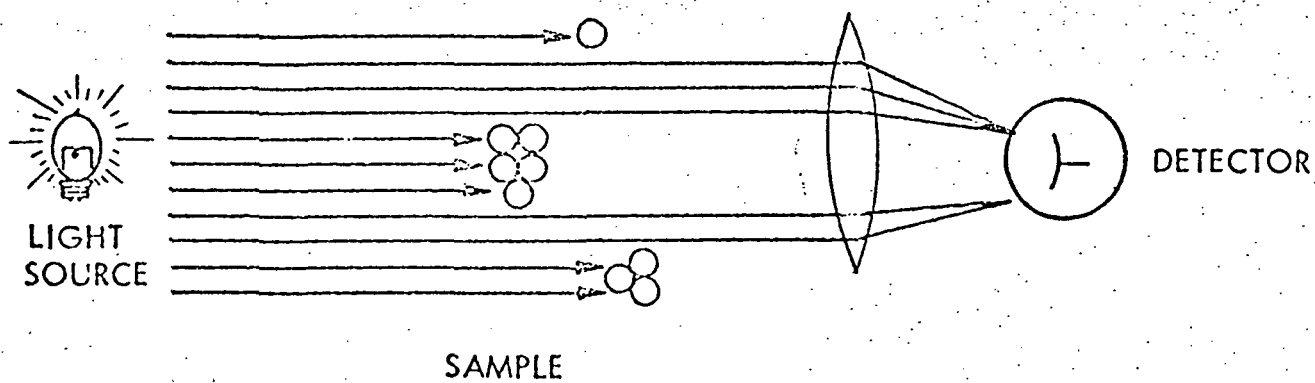
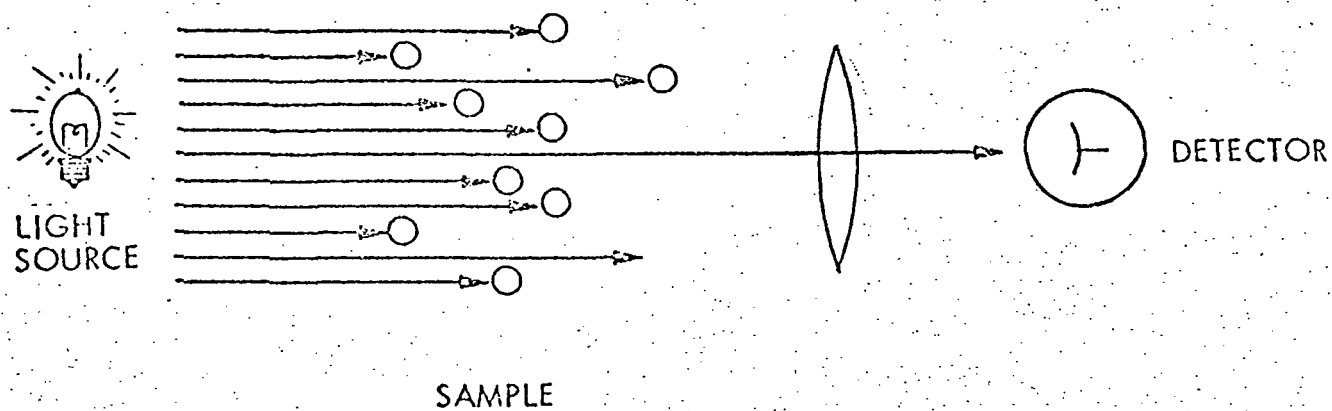


FIGURE 16

## EQUATIONS FOR OPTICAL DENSITY

UNAGGLUTINATED  
BEADS

$$\frac{D}{L} = N \left[ Q \frac{\pi}{4} d^2 \right]$$

AGGLUTINATED  
BEADS

$$\frac{D}{L} = N \left[ Q \frac{\pi}{4} d^2 \right] \frac{1}{F^{2/3} N_b^{1/3}}$$

$D/L$

OPTICAL DENSITY PER UNIT PATH LENGTH

$N$

BEAD CONCENTRATION (BEADS/CM<sup>3</sup>)

$d$

BEAD DIAMETER

$Q$

EXTINCTION COEFFICIENT (3.6 MAX. FOR  
POLYSTYRENE BEADS IN WATER)

$F$

PACKING FACTOR (TYPICALLY 0.7)

$N_b$

MEAN CLUMP SIZE (BEADS/CLUMP)

FIGURE 17

ABSORPTION OF LIGHT WITH  $0.8\mu$  NaCl AGGLUTINATED BEADS

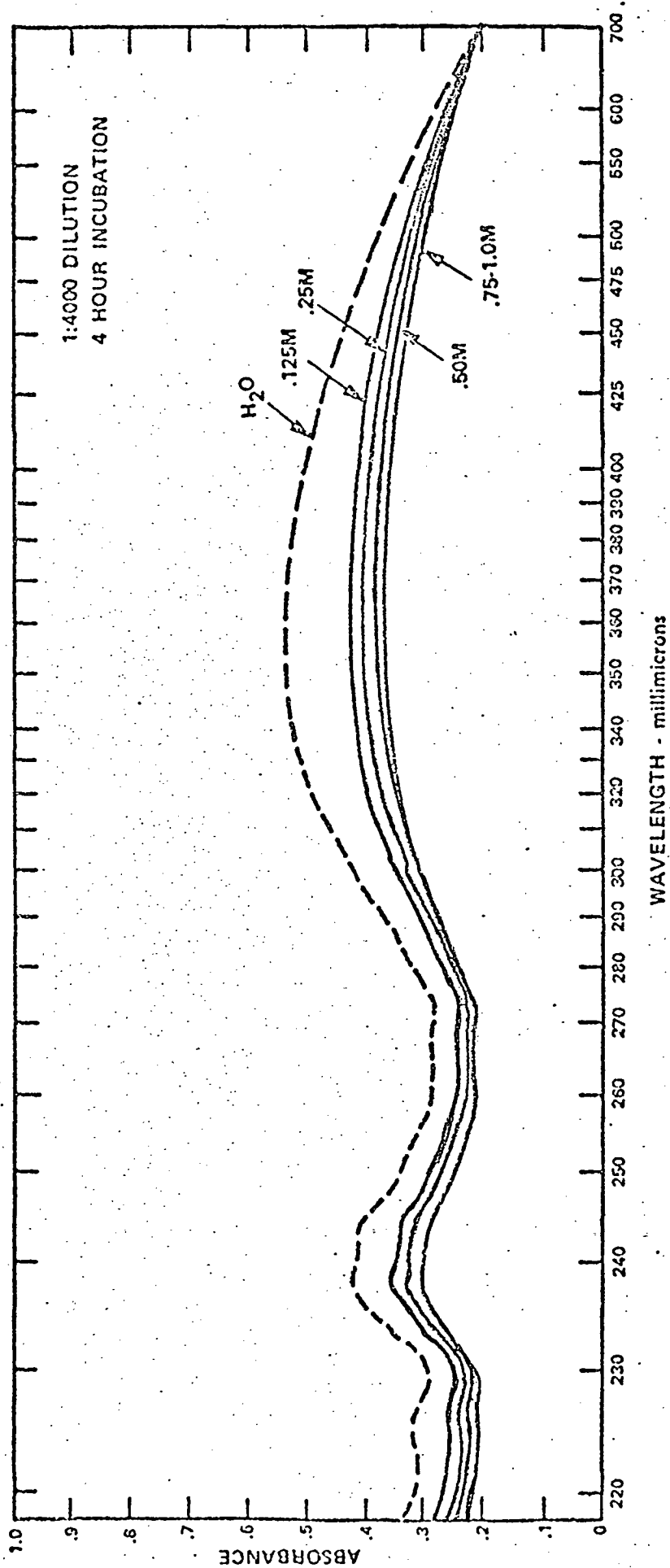
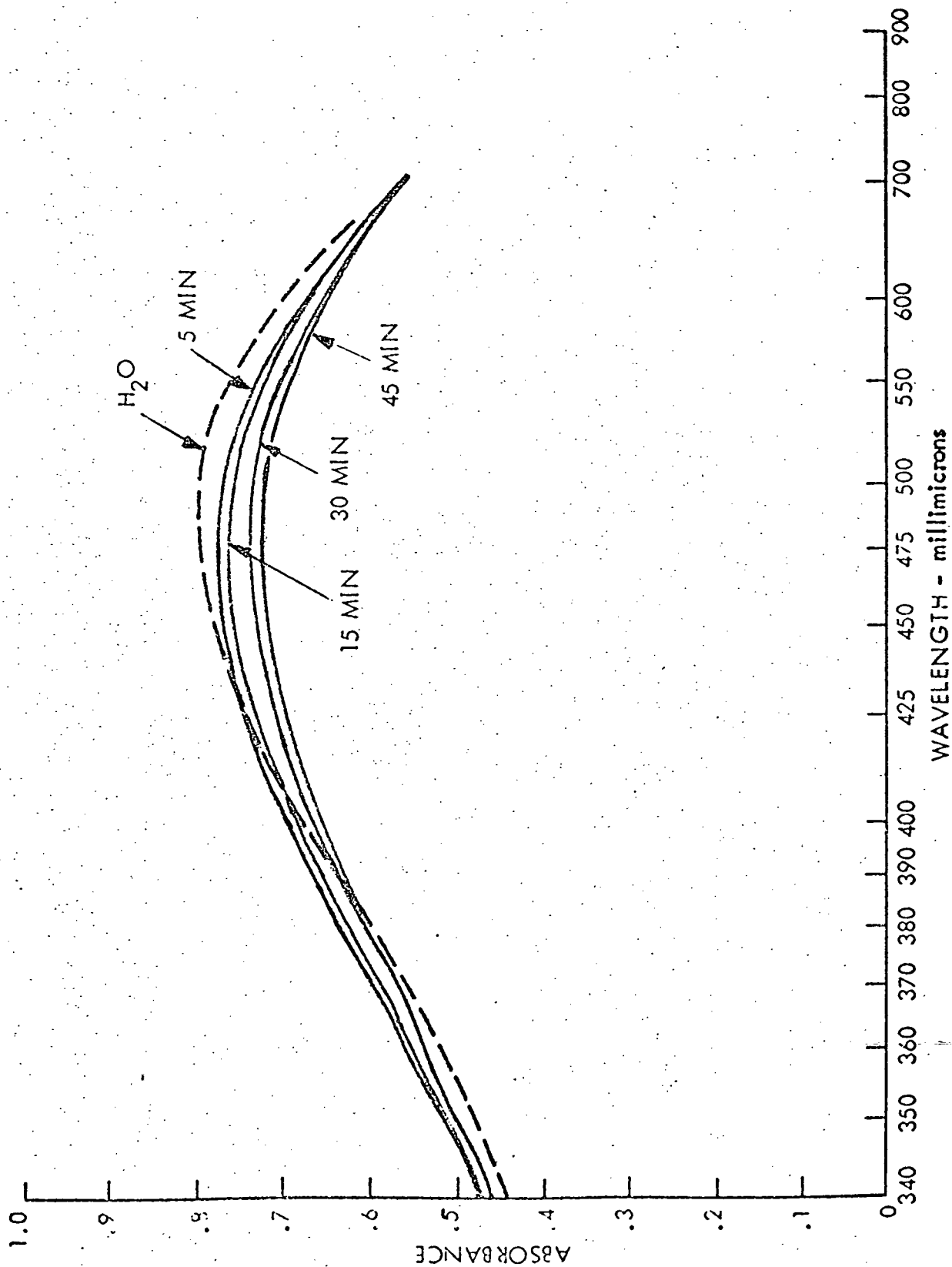




FIGURE 18

ABSORPTION OF VISIBLE LIGHT WITH  $1.1\mu$  NaCl AGGLUTINATED BEADS



region 0.45 - 0.48  $\mu$  (0.55  $\mu$  theoretical). Figure 17 shows the erratic oscillations in the UV and a well behaved characteristic in the visible wavelengths. Also shown (Figures 17 and 18) is the effect of agglutination. Increasing the reaction time or the antigen concentration (in Figure 17 the antigen is NaCl which causes clumping) reduces the light absorbance or increases light transmission. It is apparent that photometric measurements should be confined to the visible light portion of the spectrum where absorption is well behaved. Also, the effects of agglutination are most pronounced in the visible spectrum near the absorbance peak.

For comparison of data obtained from the DK-2 with that obtained with the celloscope, 1.1  $\mu$  size beads were used. The Celloscope instrument settings (gain and amplification) had to be adjusted to read the smaller particles on the 48  $\mu$  orifice, so a comparison with the normal Celloscope system was not attempted. In the comparison of the Celloscope to the light attenuation system, the samples were processed identically up to the readout, where the samples for the Celloscope were additionally diluted. As a general rule, the ratio of total beads in the light attenuation versus Celloscope is 1:100. As with the Celloscope, similar results from run to run (DK2-A) are dependent on equivalent sample conditions. In addition, the concentration of beads in the sample was shown to appreciably affect the results.

A test series was performed using both varied concentrations in the PIA reaction and varied dilutions of this reacted suspension for photometric analysis. An optimum final bead dilution (1:2000) of the stock suspension was selected for the photometric readout. The following data is based on the S-N of the difference at 475 nm - 775 nm, i.e., (Abs. 475 nm - Abs. 775 nm) sample - (Abs. 475 nm - Abs. 775 nm) control.

| <u>HA/ml</u> | <u>1:5000</u> | <u>1:4000</u> | <u>1:3000</u> | <u>1:2000</u> | <u>1:1000</u> |
|--------------|---------------|---------------|---------------|---------------|---------------|
| 0.256        | 53            | 45            | 38            | 68            | off scale     |
| 0.078        | 17            | 6             | 20            | 20            | off scale     |

Table 17 shows the effect of the reaction concentration on virus specific signals. The dilutions indicated in Table 17 (100-900) correspond to the degree of dilution of the sensitized bead reagent from

TABLE 17  
MYXOVIRUS SENSITIZED BEAD REACTION--  
CONCENTRATION STUDIES

| Antigen<br>Concentration<br>HA/ml | Dilution from Stock Beads |             |             |             |             |
|-----------------------------------|---------------------------|-------------|-------------|-------------|-------------|
|                                   | 100                       | 300         | 500         | 700         | 900         |
| 0.256                             | .120*                     | .228        | .214        | .237        | .232        |
| 0.077                             | .026                      | .065        | .047        | .103        | .066        |
| 0.077                             | .014                      | .072        | .182        | .173        | .156        |
| 0.077                             | .010                      | .036        | .029        | .069        | .067        |
| 0.077                             | <u>.019</u>               | <u>.068</u> | <u>.102</u> | <u>.135</u> | <u>.133</u> |
| Average                           | .017                      | .060        | .065        | .095        | .081        |

\*Virus specific signal

the stock bead concentration. The sensitized beads were reacted with the antigen concentration for one hour at 40 C prior to centrifugation at 3000 rpm. After concentration, the beads were then reacted an additional hour at ambient temperature before being reconstituted to test. These solutions were diluted further to 1:2000 of stock and read on the DK-2 spectrophotometer. The optimum dilution for the reaction in nearly all cases, occurs at a 1:700 dilution of the stock beads.

In the comparison of the two readout methods, a specific statistic for the DK-2 was selected to quantitatively relate the two methods. The absorption characteristics were measured throughout the visible range. Since the absorption maxima for 1  $\mu$  beads is at 475 nm and absorption at 775 nm changes very little with agglutination, the absorption was recorded at those two wavelengths and the difference was calculated. In a sample which is monodisperse, the light absorption differential is maximized between the two wavelengths. In a sample which is clumped the scattering (absorption) occurs to a limited extent and the difference between the two wavelengths is at a minimum. For comparative purposes,

difference in absorbance at the two wavelengths was subtracted from 1.00 so that both the Celloscope and DK-2 gave a higher reading with increasing antigen concentration.

In the comparison tests (with a wide range of experimental conditions) were averaged and evaluated by two methods, linear regression analysis and the paired sample "t" test. In Table 18, data are presented for the percent reduction in signal as antigen concentration (in the reaction) is decreased. These data are graphically depicted in Figure 19 and are described linearly by taking the log of the % reduction in signal as shown in Figure 20. The correlation coefficients for the DK-2 and Celloscope were 0.97 and 0.99, respectively, and indicated a strong linear relationship. The same conditions were utilized for readout methods, so that the difference in the sharpness of the slope may be attributed to the nature of the readout method. It appeared that larger signal differences were observed more often with the Celloscope.

TABLE 18  
COMPARISON OF DK-2 AND CELLOSCOPE  
READOUT METHODS

| Antigen<br>Concentration<br>(EID <sub>50</sub> /ml) | Celloscope        |                       | DK-2              |                       |
|---|-------------------|-----------------------|-------------------|-----------------------|
|   | Percent<br>Signal | Log Percent<br>Signal | Percent<br>Signal | Log Percent<br>Signal |
| 10 <sup>6</sup>                                     | 100               | 2.0                   | 100               | 2.00                  |
| 5 x 10 <sup>5</sup>                                 | 59                | 1.77                  | 95.9              | 1.98                  |
| 10 <sup>5</sup>                                     | 16.9              | 1.23                  | 66.9              | 1.83                  |
| 5 x 10 <sup>4</sup>                                 | 4.9               | 0.69                  | 54.7              | 1.74                  |
| 10 <sup>4</sup>                                     | 3.1               | 0.49                  | 50.9              | 1.71                  |
| 5 x 10 <sup>3</sup>                                 | 1.0               | 0.00                  | 47.7              | 1.68                  |

FIGURE 19  
EFFECT OF DECREASING VIRAL ANTIGEN CONCENTRATION  
ON PERCENT SIGNAL REDUCTION WITH THE DK-2  
AND CELLOSCOPE READOUT METHODS

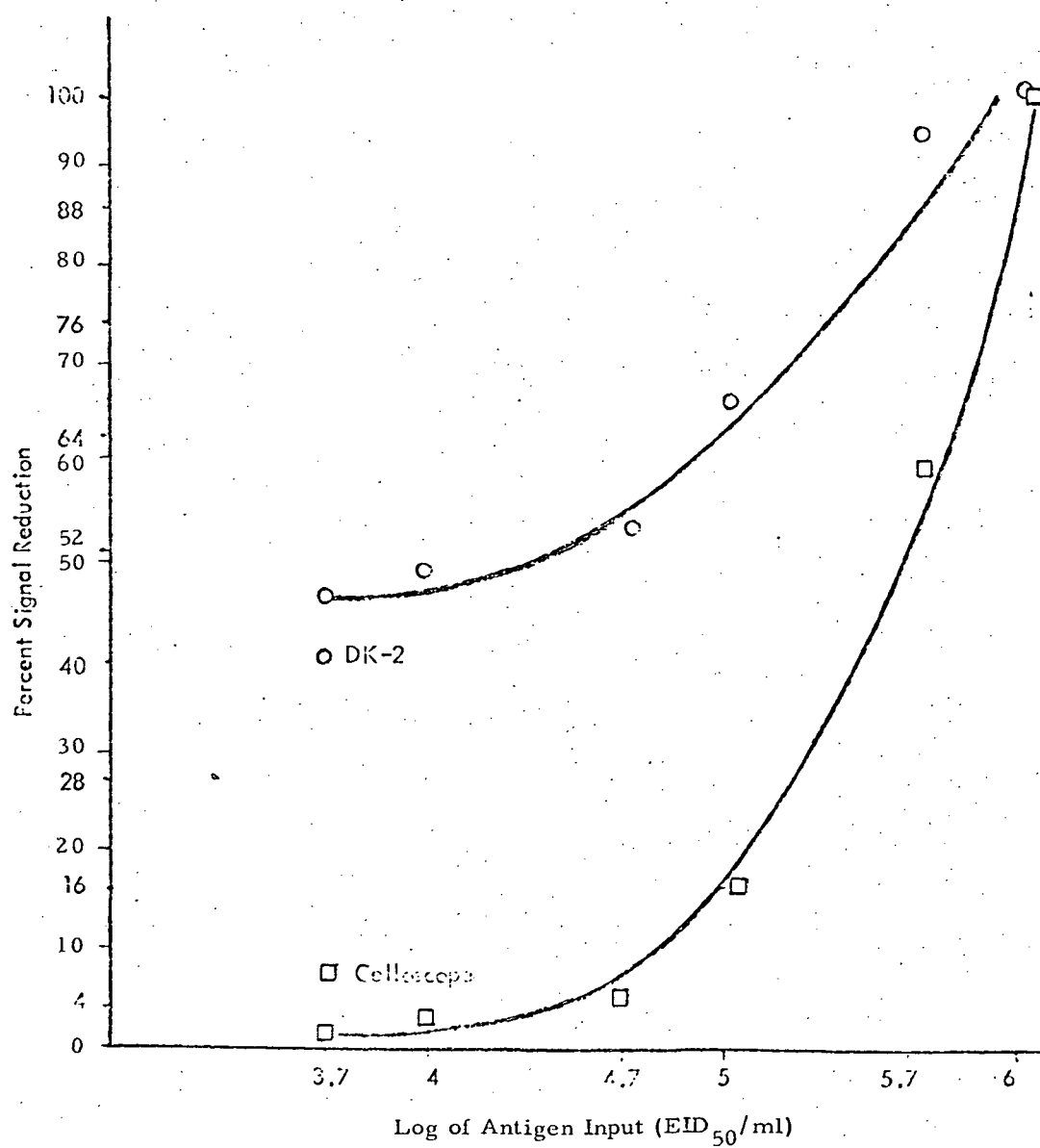
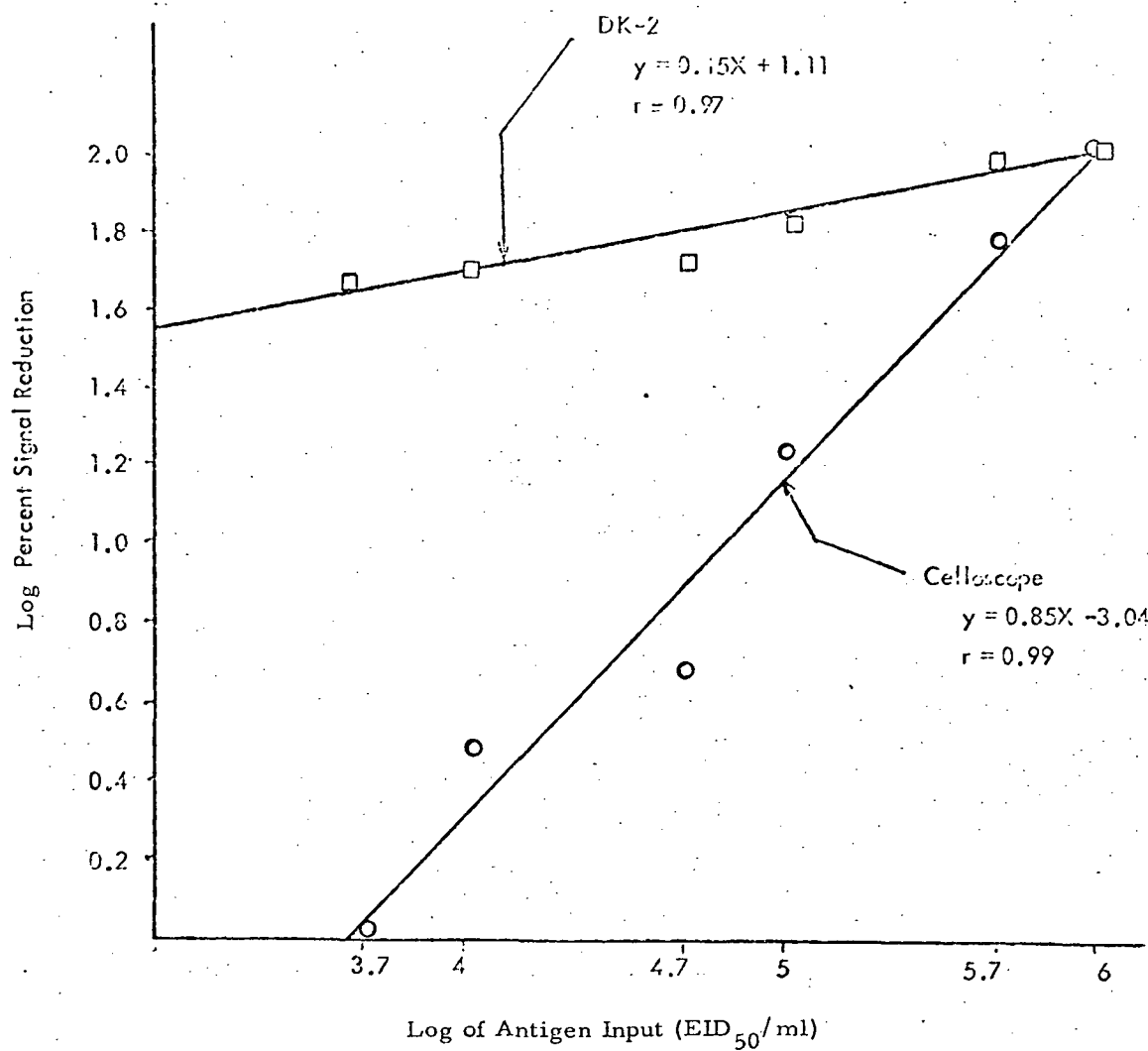


FIGURE 20  
EFFECT OF DECREASING VIRAL ANTIGEN CONCENTRATION  
ON PERCENT SIGNAL REDUCTION WITH THE DK-2  
AND CELLOSCOPE READOUT METHODS



Since the slope was 7 times smaller with DK-2, one might be concerned with the significance of the signal difference between narrow antigen increments. To test this condition, the paired sample "t" test was performed on both the Celloscope and DK-2 data. The results are expressed in Table 19. From this table the comparison indicates that both readout methods detected a given concentration with approximately the same confidence. Two exceptions  $10^6$  vs  $5 \times 10^5$  and  $10^5$  vs  $5 \times 10^4$  were evident, and the reason for these variances is not known, but separation at either level was not considered critical to the use of the readout. In all cases, both methods sensed a higher signal for increasing antigen concentration. In conclusion, both methods (70% confidence or greater) distinguished  $10^4$  from  $5 \times 10^3$ ,  $5 \times 10^4$  from  $10^4$ ,  $5 \times 10^5$  from  $10^5$ ,  $10^5$  from  $10^4$  and  $10^6$  from  $10^5$ .

TABLE 19  
COMPARISON OF THE PROBABILITY OF SIGNIFICANT SIGNAL  
DIFFERENCES OF NARROW MYXOVIRUS CONCENTRATIONS  
WITH THE CELLOSCOPE AND DK-2 READOUT METHODS

DK-2

| Antigen<br>Concentration<br>(EID <sub>50</sub> /ml) | t statistic | Degrees of<br>Freedom | % Confidence or Probability<br>of Difference |
|---|-------------|-----------------------|--|
| 5 x 10 <sup>3</sup> vs 10 <sup>3</sup>              | 0.76        | 7                     | 50%  |
| 10 <sup>4</sup> vs 5 x 10 <sup>3</sup>              | 1.51        | 17                    | 80%  |
| 5 x 10 <sup>4</sup> vs 10 <sup>4</sup>              | 1.15        | 10                    | 70%  |
| 10 <sup>5</sup> vs 5 x 10 <sup>4</sup>              | 1.378       | 11                    | 80%  |
| 5 x 10 <sup>5</sup> vs 10 <sup>5</sup>              | 2.05        | 12                    | 90%  |
| 10 <sup>6</sup> vs 5 x 10 <sup>5</sup>              | 0.20        | 11                    | 10%  |
| 10 <sup>5</sup> vs 10 <sup>4</sup>                  | 1.78        | 26                    | 90%  |
| 10 <sup>6</sup> vs 10 <sup>5</sup>                  | 2.25        | 27                    | 95%  |
| Celloscope  |             |                       |  |
| 5 x 10 <sup>3</sup> vs 10 <sup>3</sup>              | 0.46        | 8                     | 30%  |
| 10 <sup>4</sup> vs 5 x 10 <sup>3</sup>              | 1.76        | 9                     | 80%  |
| 5 x 10 <sup>4</sup> vs 10 <sup>4</sup>              | 1.87        | 8                     | 90%  |
| 10 <sup>5</sup> vs 5 x 10 <sup>4</sup>              | 0.64        | 9                     | 30%  |
| 5 x 10 <sup>5</sup> vs 10 <sup>5</sup>              | 1.98        | 8                     | 90%  |
| 10 <sup>6</sup> vs 5 x 10 <sup>5</sup>              | 1.22        | 7                     | 70%  |
| 10 <sup>5</sup> vs 10 <sup>4</sup>                  | 1.63        | 9                     | 80%  |
| 10 <sup>6</sup> vs 10 <sup>5</sup>                  | 2.80        | 8                     | 95%  |

\*Represents the confidence level at which the difference in signal between the designated concentrations is not due to sampling error.



## SECTION 4

4.0

### BREADBOARD DESIGN, FABRICATION AND EVALUATION

The photometric system for the detection of agglutination was chosen for further evaluation and development of a test bed. The photometric system is relatively simple and a large body of experience in the design of such instruments is available. No change needs to be made in reagents or in the reaction process. The system does not involve pumping the bead suspension through small orifices and thus avoids the problems of orifice plugging or disruption of clumps inherent in the Celloscope system. The primary phenomenon involved in the measurement of the agglutination of the latex beads is light scattering. This may be measured directly by measurement of the scattered light or indirectly by measurement of the attenuation of transmitted light.

Qualitatively, the optical transmission of agglutinated bead solutions behaves as follows: The individual beads scatter and absorb light with scattering dominating. At wavelengths comparable to the bead radius or a little longer, Mie scattering is at a maximum, and, hence, the transmission is at a minimum. With greater agglutination, the beads tend more to shield each other, so the probability of light attenuation is reduced and the transmission is increased. However, at wavelengths long compared to the particle diameter, Rayleigh scattering is dominant, which is proportional to  $A^6 \lambda^{-4}$  ( $A$  is particle radius;  $\lambda$  is light wavelength). The light will tend not to resolve the individual beads of a cluster, but will see a cluster more as a single particle of much larger mean radius. Now greater agglutination increases the likelihood of scattering faster than it decreases the number of scattering particles. The scattering should be proportional to the mean number of beads in a cluster. Thus, greater agglutination will decrease the transmission of long wavelength light.

A semi-quantitative preliminary analysis is possible. For the initial unagglutinated suspension ( $N_b = 1$  bead/clump), the absorption cross-section (actually a measure of the scattering or absorption probability) is proportional to the number of particles, the geometric cross-section per particle, and the extinction coefficient deduced from Mie theory. Thus, the optical density,  $D$ , of unagglutinated beads is

$$D = \alpha L \simeq \frac{C}{N_b} \frac{\pi}{4} d^2 Q L \quad (1)$$

where

$\alpha$  is linear extinction coefficient ( $\text{cm}^{-1}$ )

$L$  is optical path length (cm)

$C$  is bead concentration (beads/ $\text{cm}^3$ )

d is bead diameter (cm)

Q is extinction coefficient dimensionless)\*

$N_b$  is number of beads/clump (=1 for unagglutinated samples)

For agglutinated solutions,

$$D = \alpha L \approx N_c \frac{\pi}{4} d_c^2 Q L \quad (2)$$

$$N_c = \frac{C}{N_b}$$

$$d_c = d_o \left[ \frac{N_o}{N_c} \right]^{1/3}$$

Here,  $N_c$  is the clump concentration (clumps/cm<sup>3</sup>), and  $d_c$  is now the mean diameter of a clump.

For .8  $\mu$  beads at reaction concentration ( $\sim 7 \times 10^7$  beads/cm<sup>3</sup>) from equation (1),

$$D = (7 \times 10^7) \frac{\pi}{4} (.8 \times 10^{-4})^2 (2.0)(1) = .704$$

If the above sample is now agglutinated so that there are an average of 2.3 beads per clump, then for the control, from equation (2),

$$N_c = \frac{7 \times 10^7}{2.3} = 3.04 \times 10^7 \text{ clumps/cm}^3$$

and

$$d_c = .8 \times 10^{-4} \left[ \frac{7 \times 10^7}{3.04 \times 10^7} \right]^{1/3} = 1.055 \times 10^{-4} \text{ cm.}$$

Then,

$$D = (3.04 \times 10^7) \frac{\pi}{4} (1.055 \times 10^{-4})^2 (2)(1) = .531$$

If virus is present, the average clump size has been experimentally determined to be  $\sim 2.7$  beads per clump. Then,

$$N_c = \frac{7 \times 10^7}{2.7} = 2.5 \times 10^7 \text{ clumps/cm}^3$$

and

$$d_c = .8 \times 10^{-4} \left[ \frac{7 \times 10^7}{2.5 \times 10^7} \right]^{1/3} = 1.11 \times 10^{-4} \text{ cm.}$$

Then,

$$D = (2.5 \times 10^7) \frac{\pi}{4} (1.11 \times 10^{-4})^2 (2)(1) = .485$$

\* The value of the extinction coefficient, Q, is estimated from data presented in Handbook on Aerosols, Chapter 7, AEC Report STR-10-1, 1963

Transmission of the sample is related to optical density as follows:

$$\mathcal{T} = 10^{-D} = 10^{-\alpha L} \quad (3)$$

The change in optical density from .531 to .485 equates to a transmission change from 29.4% to 32.8%. On the basis of the above calculation, it may be concluded that the photometric approach should offer an accurate means of measuring the degree of agglutination.

Based upon the above principles of the photometric theory, it is possible to enumerate those characteristics which the photometer should have (Figure 21).

1. The system should have high sensitivity to agglutination so that small differences between sample and control can be detected.
2. The photometer should have low sensitivity to bead size. This also means that system operation should be confined to the visible light spectrum where absorption characteristics are well behaved.
3. Selection of  $1.1\mu$  beads was made because it permits operation in the visible spectrum (peak absorption occurs at a wavelength of about  $1/2$  the particle diameter) and because conductometric data was available for comparison.
4. A 1 cm path length was selected simply because it permits the use of standard spectrometer cuvettes.
5. An optical density near 1.0 was selected based upon experience in making sample preps. It was somewhat arbitrary although it was convenient and was typical of the preparations used at AMB.
6. Once the selection of bead size was made, the wave band for agglutination measurements was dictated by the photometric theory to be  $\sim 1/2$  the bead diameter or about 550 nm (in actual practice the peak absorption for  $1.1\mu$  beads occurred at about 450 nm).
7. Similarly, after the bead size and optical density had been selected, the theory defined the bead concentration to be in the range of  $3 - 5 \times 10^7$  bead/cm<sup>3</sup>.

FIGURE 21

## SAMPLE DESIGN

### DESIGN REQUIREMENTS:

- High Sensitivity to Agglutination
- Low Sensitivity to Bead Size

### SELECTED PARAMETERS

- $1.1 \mu$  particles
- 1 cm path length
- $\sim 1.0$  optical density

### CALCULATED PARAMETERS

- 450 m  $\mu$  wavelength
- $3 - 5 \times 10^7$  bead/cm<sup>3</sup>

#### 4.1 SYSTEM DESCRIPTION

A photometric test bed was designed and fabricated for making optical measurements of agglutinated bead samples. A schematic of the optical paths is shown in Figure 22. Collimated light is passed through a bandpass filter. This filter is a circular variable filter having 25 nanometer bandwidths adjustable over the range from 400 to 700 nm. The incoming light is split into two beams, passed through a beam chopper, through the sample and reference cuvettes, is recombined and sent to a photomultiplier tube.

Dual beams are provided to accommodate "simultaneous" readout of a sample and a control. Because of nonspecific agglutination characteristics anticipated to occur with each astronaut, it is expected that a "control" will have to be prepared for each astronaut sample. Since agglutination progresses with time, it is necessary to read both sample and control at the same time.

A single lamp and detector are used to avoid problems relating to non-uniformities in light output and detector degradation. Both light beams are modulated by the chopper at 10 cps in order that the processing electronics can discriminate between the sample and control. The modulating frequency is selected on the basis of the response characteristics of the related electronics. The beam modulation (chopping) also serves to switch between sample and control cuvettes.

The processing electronics will monitor the sample or control beams by means of light emitting diodes and detectors which will be modulated (chopped) with the beam chopper. The resulting detector (PMT) signals will be electrically divided and displayed on a digital readout as a ratio of sample signal-to-control signal.

The photometer is capable of monitoring either direct transmitted energy or energy which is scattered at 90 degrees. Shutters are provided for selection of energy type.

#### 4.2 SYSTEM SENSITIVITY

Based on the photometric theory and selected system parameters, the sensitivity of the photometric method toward detection of agglutination was evaluated and is presented in Figure 23.

FIGURE 22

# MEMS PHOTOMETER OPTICAL PATH

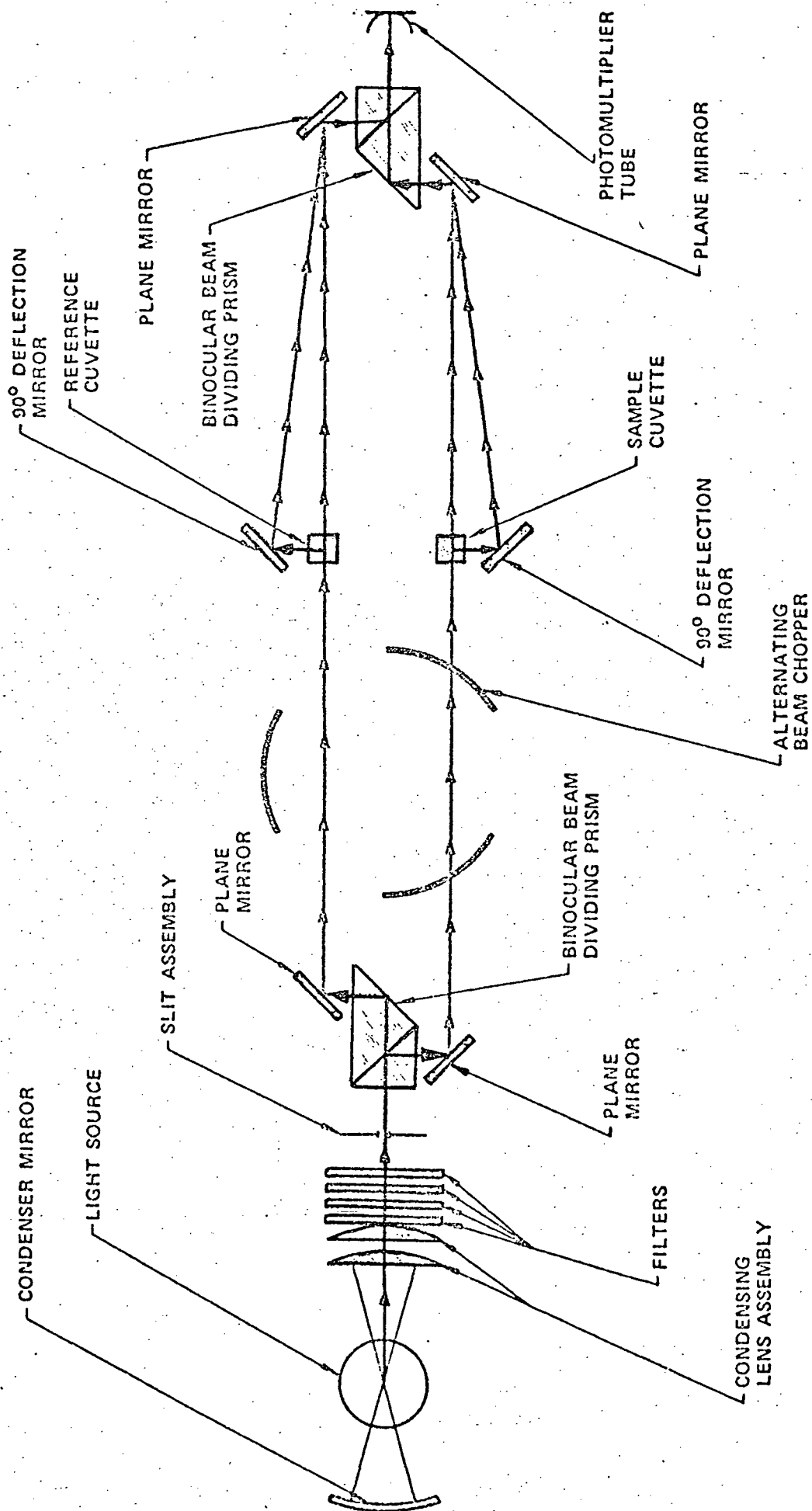


FIGURE 23

# SENSITIVITY TO AGGLUTINATION

$$N \left[ Q \frac{\pi}{4} d^2 \right] \frac{L}{F^{2/3}} \left( \frac{1}{N_b^{1/3}} \right) = D$$

For Controls:

$$4 \times 10^7 \left[ 3.6 \frac{\pi}{4} (1.1 \times 10^{-4})^2 \right] \frac{1}{(0.7)^{2/3}} \left( \frac{1}{2.0^{1/3}} \right) = 1.38$$

For Sample:

$$4 \times 10^7 \left[ 3.6 \frac{\pi}{4} (1.1 \times 10^{-4})^2 \right] \frac{1}{(0.7)^{2/3}} \left( \frac{1}{2.4^{1/3}} \right) = \frac{1.30}{.08}$$

Both the control and the sample solutions contain  $1.1\mu$  beads with a concentration of  $4 \times 10^7$  beads/cm<sup>3</sup>. The packing factor for both is about 0.7. Microscopic examination of agglutinated solutions has established that a control will typically average about 2.0 beads per clump. A sample may have an infection level low enough that agglutination results in an average clump size of about 2.4 beads/clump. Insertion of these numbers in the equation for optical density gives an O.D. difference between sample and control of 0.08. Examination of data obtained from the photometer showed that an O.D. change of about .02 is easily readable (electronically differences of .001 may be read). In conclusion the photometer sensitivity for detection of differences between sample and control is at least 4 times better than the levels likely to be experienced.



#### 4.3 PHOTOMETRIC TEST BED EVALUATION

Biological evaluation of the test bed was carried out using  $1.1\mu$  latex beads sensitized with influenza antibody. Preliminary photometric analysis of the reacted sensitized beads was performed on a Beckman DK-2 photometer and involved making comparative absorbance readings between a buffer blank and the reacted beads at two wavelengths - 700 nm and 475 nm. The capabilities of the test bed photometer permitted modification and simplification of these parameters.

The test bed photometer was tested with influenza sensitized  $1.1\mu$  beads at varied antigen concentrations. These reacted suspensions were read on the photometer against either a buffer blank (no beads) or the control suspension of sensitized beads with no antigen present. These were measured at 475 nm only. Table 20 shows a comparison of the data obtained by the two methods. The use of sensitized beads without antigen as a control was chosen as the readout mode. The data, graphically shown in Figure 24, demonstrates that the signal to noise ratio is greater using this method. In addition, less readings are required and the correction for blank is made automatically.

Two bead sizes were also compared using the photometric test bed. Since  $2.0\mu$  latex beads were generally used on conductometric testing, they were tested photometrically in comparison with the  $1.1\mu$  latex beads. An optimum wavelength of 700 nm was chosen for reading the reacted  $2.0\mu$  latex beads. The data depicted in Figure 25 demonstrates excellent linearity of the results. Correlation coefficients were determined for the log/log relationship and found to be 0.97 and 0.99, respectively for the  $1.1\mu$  and  $2.0\mu$  beads. The  $1.1\mu$  latex beads showed a somewhat increased sensitivity and higher signal than did the  $2.0\mu$  latex beads, so  $1.1\mu$  beads were used in subsequent testing.

A single wavelength was chosen at which to accumulate the reaction readouts. There was experimentally no gain in sensitivity or reliability by using a difference between two wavelengths. An optimum wavelength of 550 nm was predicted for  $1.1\mu$  latex beads on a theoretical basis. The experimental peak using  $1.1\mu$  beads actually appeared at 475 nm.

TABLE 20

COMPARISON OF PHOTOMETER CONTROL SYSTEMS  
1.1 $\mu$  MYXOVIRUS SENSITIZED BEADS

| Hemagglutinating<br>Units per ml | Bead Control          |      | Buffer Control        |      |
|----------------------------------|-----------------------|------|-----------------------|------|
|                                  | Photometer<br>Reading | Log  | Photometer<br>Reading | Log  |
| .026                             | 155                   | 2.19 | 152                   | 2.18 |
| .128                             | 153                   | 2.18 | 148                   | 2.17 |
| .256                             | 259                   | 2.41 | 168                   | 2.23 |
| 1.28                             | 324                   | 2.51 | 216                   | 2.33 |
| 2.56                             | 527                   | 2.72 | 263                   | 2.42 |
| 12.8                             | 619                   | 2.79 | 339                   | 2.53 |

FIGURE 24  
COMPARISON OF PHOTOMETER CONTROL SYSTEMS  
1.1 $\mu$  MYXOVIRUS SENSITIZED BEADS

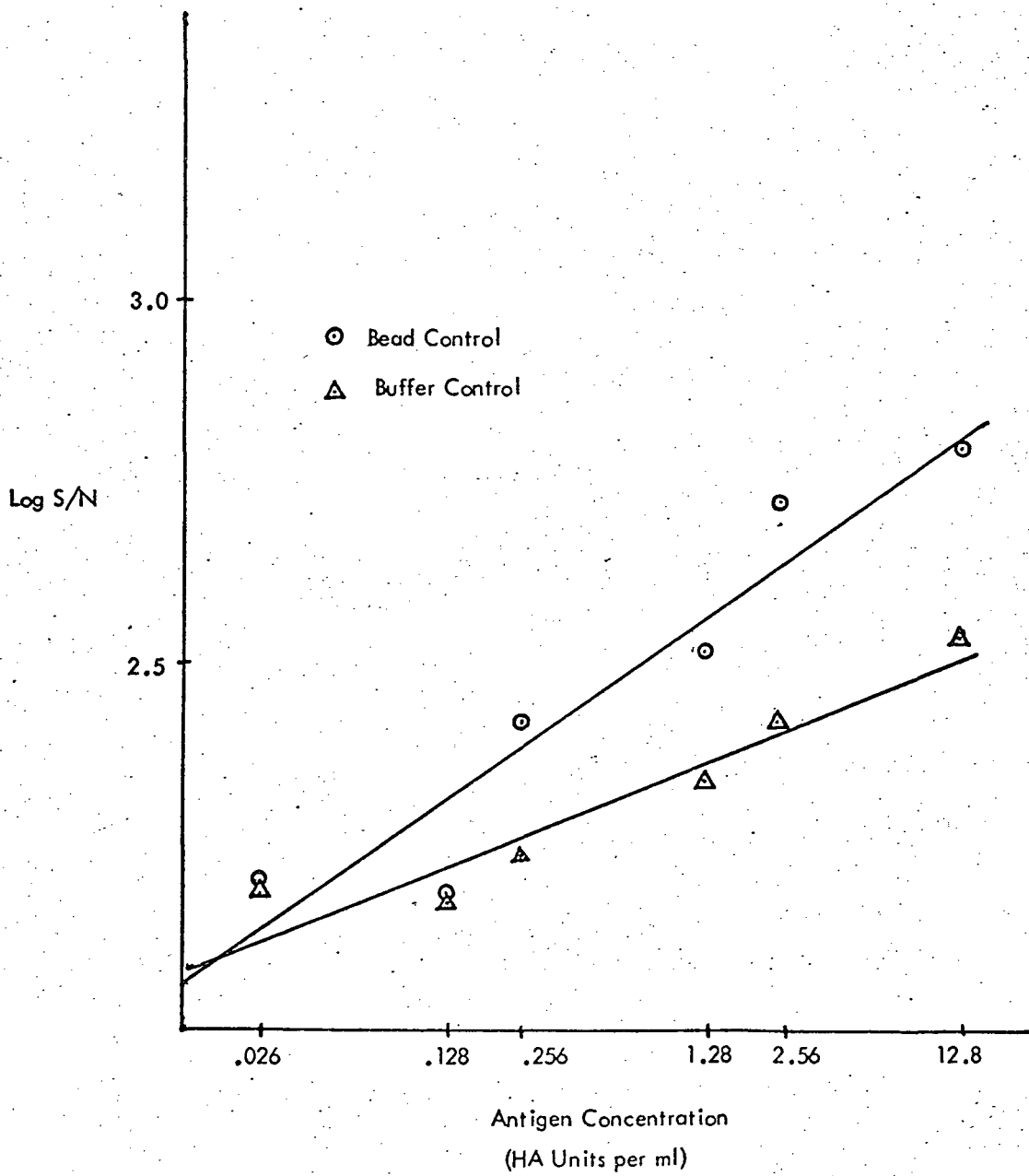
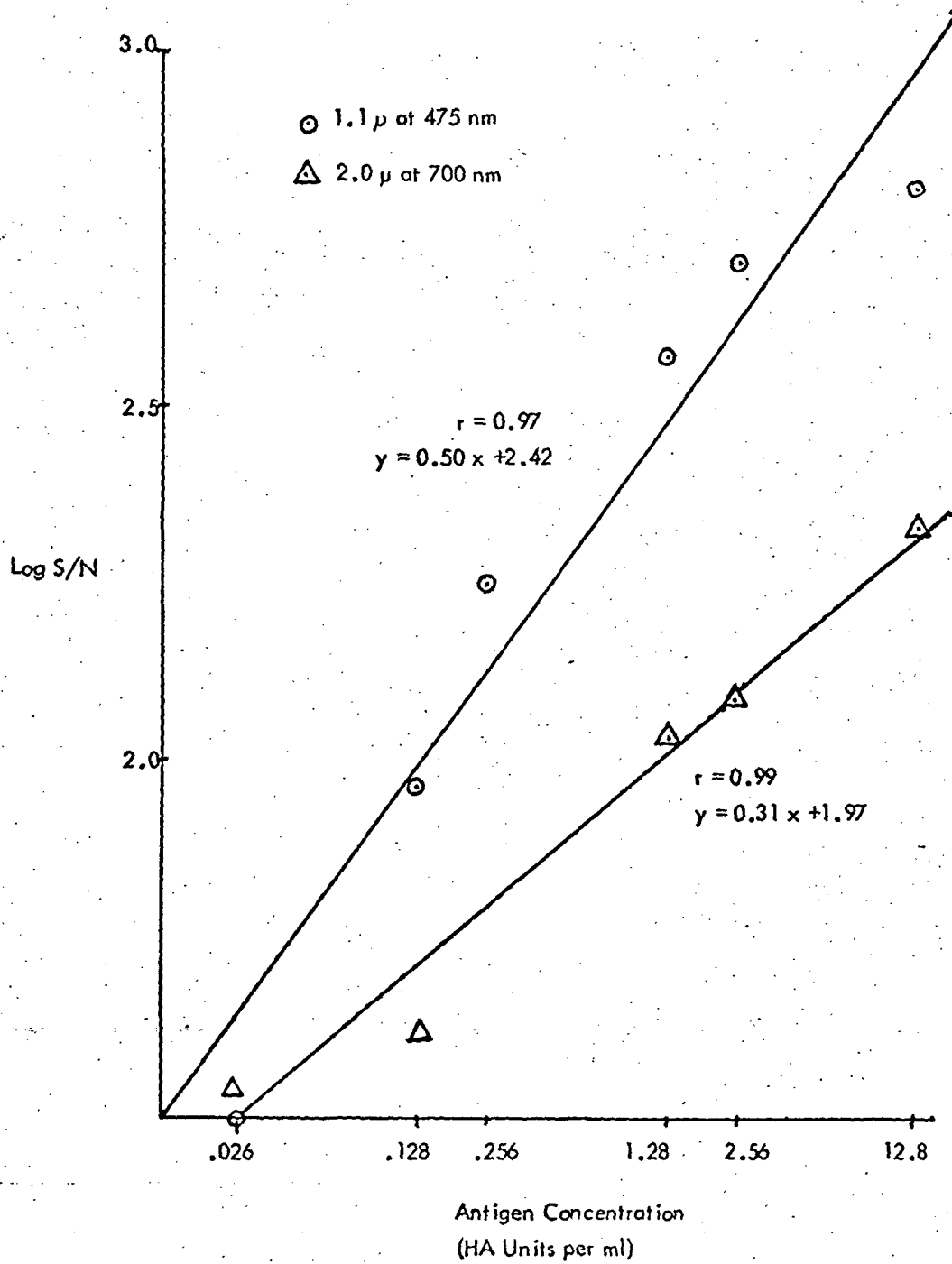


FIGURE 25  
 BEAD SIZE COMPARISON PHOTOMETER  
 MYXOVIRUS SENSITIZED 1.1 $\mu$  AND 2.0 $\mu$  LATEX BEADS



The photometric test bed evaluation was accomplished using a single wavelength setting (475 nm) comparing influenza antibody sensitized beads with no antigen against the same beads in the presence of serially diluted influenza antigen. These PIA reactions were prepared and simultaneously readout on both test beds - conductrometric and photometric. This comparative data shown on Table 21 from the conductrometric test bed and on Table 22 from the photometric test bed was compiled from 3 to 6 test series for each point and at two reaction times - one hour and 18 hours. The data points for 1 hour reaction of influenza antibody sensitized 1.1  $\mu$ m latex beads with serially diluted influenza antigen are visually integrated into Figure 26. The data points at 18 hours of reaction are graphically shown in Figure 27.

It will be noted that in all cases the response curve was generally sigmoid in shape. This was most pronounced at the longer reaction times. At the low end of the scale increasing antigen concentration had little effect on S/N. The point at which a positive dose/response relationship was attained for the 1 hour reaction was considerably lower for the conductrometric system than for the photometric system. However, after 18 hours of reaction the results were more comparable for the two systems.

Experimentally, the photometric test bed appeared to have somewhat less sensitivity than the conductrometric system. Further optimization of the photometric system should increase the signal or decrease the noise which would result in sensitivities comparable to the conductrometric system.

TABLE 21  
CONDUCTOMETRIC DOSE RESPONSE

| Antigen<br>Concentration<br>HA Units/ml | Avg S/N |        | Log S/N |        |
|---|---------|--------|---------|--------|
|   | 1 Hr    | 18 Hrs | 1 Hr    | 18 Hrs |
| .003                                    | 26.4    | 53.7   | 1.42    | 1.73   |
| .026                                    | 22.9    | 70.0   | 1.36    | 1.85   |
| .128                                    | 29.2    | 69.2   | 1.47    | 1.84   |
| .256                                    | 47.4    | 188.3  | 1.68    | 2.27   |
| 1.28                                    | 120.6   | 3697   | 2.08    | 3.57   |
| 2.56                                    | 474.9   | 6506   | 2.68    | 3.81   |
| 12.8                                    | 2347    | 12000  | 3.37    | 4.08   |

TABLE 22

## PHOTOMETRIC DOSE RESPONSE

| Antigen<br>Concentration<br>HA Units/ml | Avg S/N |        | Log S/N |        |
|---|---------|--------|---------|--------|
|   | 1 Hr    | 18 Hrs | 1 Hr    | 18 Hrs |
| .003                                    | -       | 63     | -       | 1.80   |
| .026                                    | 91      | 31     | 1.96    | 1.49   |
| .128                                    | 93      | 93     | 1.97    | 1.97   |
| .256                                    | 72      | 179    | 1.86    | 2.25   |
| 1.28                                    | 211     | 375    | 2.32    | 2.57   |
| 2.56                                    | 260     | 506    | 2.41    | 2.70   |
| 12.8                                    | 441     | 641    | 2.64    | 2.81   |

FIGURE 26  
DOSE RESPONSE RELATIONSHIP  
FOR  
CONDUCTOMETRIC AND PHOTOMETRIC MEASUREMENTS  
One Hour Reaction

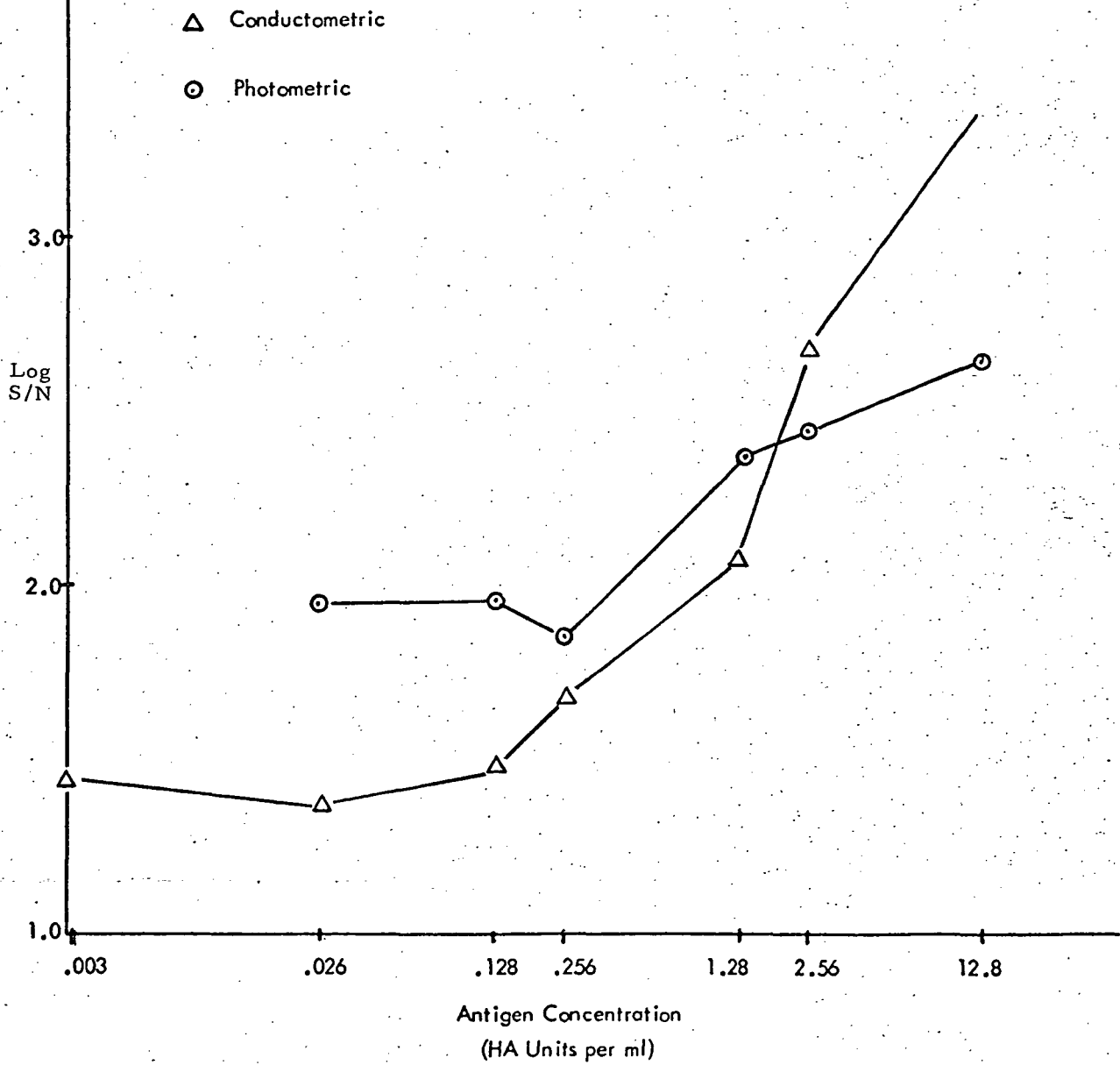
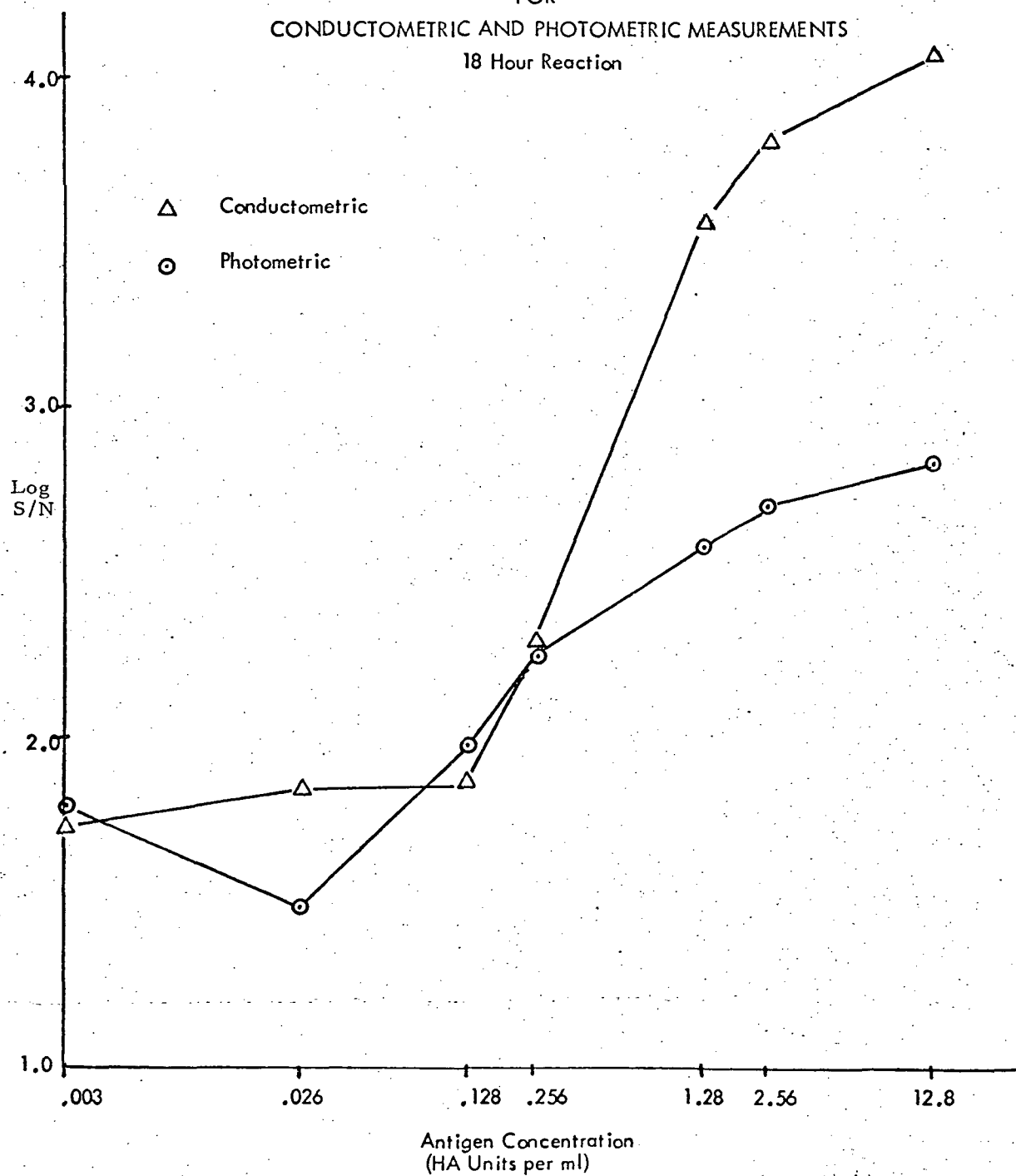




FIGURE 27  
DOSE RESPONSE RELATIONSHIP  
FOR  
CONDUCTOMETRIC AND PHOTOMETRIC MEASUREMENTS  
18 Hour Reaction



## SECTION 5

5.0

### OTHER PIA APPLICATIONS

Detection of microorganisms using sensitized beads is a rapid and sensitive method as has been shown with the influenza virus and Mycoplasma pneumoniae. The use of this method for detection or identification of bacteria might eliminate the need for the time consuming procedures of culturing, sub-culturing and the many biochemical tests usually performed to identify a suspected pathogenic bacterium. Initial isolation of the organism probably would still be necessary in order to have a pure culture. But even if an initial isolation by conventional means were necessary, much time would be saved in identifying the bacteria in question.

Applications of the PIA method for detection of microorganisms was expanded to include the feasibility of bacterial detection using the group A Streptococcus and for group virus detection using adenovirus.

The detection and identification of individual viruses from a clinical sample would be expensive and time consuming because of the many virus groups and numerous types within some groups. One approach to eliminate this problem would be the detection of virus groups. Group detection would offer the clinician sufficient identification in most cases. The use of sensitized beads can be applied to the detection of group specific antigens.

Certain groups of viruses such as the adenoviruses have antigens that are common to all the members of the group. If this group antigen is isolated, purified and concentrated, antibody can be produced to it. The resultant sensitized beads should then be able to detect many, if not all, members of the group providing the antigen is readily accessible.

#### 5.1 EXTENSION OF THE PIA APPROACH TO BACTERIAL DETECTION

The demonstration of the feasibility of detecting Group A Streptococcus was accomplished. The reagents (antigen and antisera) were supplied from the commercial manufacturing group at Aerojet Medical and Biological Systems. The antisera used in these studies was non-precipitating. The beads (2.02  $\mu$ ) were sensitized using the protocols established for the myxovirus and the mycoplasma. The antigen, at  $10^9$  organisms/ml, was diluted in distilled water or 0.85% NaCl and tested over a range of concentrations from  $10^0$  to  $10^9$  organisms/ml. The results, presented in Table 23, showed that  $10^9$  was significantly

TABLE 23

QUANTITATION OF THE LATEX AGGLUTINATION TEST FOR  
STREPTOCOCCUS GROUP A WITH THE CELLOSCOPE SENSOR

| Concentration<br>(organisms/ml) | Celoscope<br>Statistic                         | Signal/Noise |
|---------------------------------|--|--------------|
| $10^9$ Signal<br>Noise          | $2.05 \times 10^{12}$<br>$1.86 \times 10^{11}$ | 11.86        |
| $10^8$ Signal<br>Noise          | $1.92 \times 10^{11}$<br>$1.28 \times 10^{11}$ | 1.50         |
| $10^7$ Signal<br>Noise          | $1.04 \times 10^{11}$<br>$8.12 \times 10^{10}$ | 1.28         |
| $10^6$ Signal<br>Noise          | $9.60 \times 10^{10}$<br>$8.67 \times 10^{10}$ | 1.11         |
| $10^5$ Signal<br>Noise          | $1.14 \times 10^{11}$<br>$2.65 \times 10^{11}$ | 0.48         |
| $10^4$ Signal<br>Noise          | $3.36 \times 10^{11}$<br>$1.14 \times 10^{11}$ | 2.95         |
| $10^3$ Signal<br>Noise          | $7.98 \times 10^{10}$<br>$6.77 \times 10^{10}$ | 1.18         |
| $10^2$ Signal<br>Noise          | $1.04 \times 10^{11}$<br>$1.36 \times 10^{11}$ | 0.76         |
| $10^1$ Signal<br>Noise          | $7.47 \times 10^{10}$<br>$1.18 \times 10^{11}$ | 0.63         |
| $10^0$ Signal<br>Noise          | $7.51 \times 10^{10}$<br>$1.54 \times 10^{11}$ | 0.49         |

different (99.9% confidence) from any other concentration tested. Repeated tests substantiated these results.

To optimize the test sensitivity while confining the efforts to a feasibility study, the antigen suspension ( $10^9$  organisms/ml) was sonicated (bath-type sonic cleaner) for 30 minutes. After sonication, the cellular debris was removed by centrifugation. Twofold dilutions were prepared in distilled water. The extract (0.1 ml) was reacted with 0.1 ml of the specific sensitized bead preparation.

The results are presented in Table 24 and Figure 28. The high correlation coefficient indicated a linear relationship between the extract of the Group A Strep and the Celloscope statistic. Estimates regarding the ultimate sensitivity of the test for bacterial detection cannot be performed since the relationship between the latex reactivity of the bacterial extract versus whole organisms has not been rigorously studied. However, it is evident that sonication provided about one and one-half logs more of sensitivity ( $10^{7.5}$  vs.  $10^9$ ). Undoubtedly, using protocols specifically designed for bacterial detection (antigen and antisera pretreatments and proper reaction conditions; i.e., buffer, pH, ionic strength, temperature and time of reaction) will result in a significantly lower sensitivity threshold.

## 5.2 EXTENSION OF THE PIA APPROACH TO MULTIAGENT DETECTION

The surface of the virion of adenovirus is composed of 252 capsomeres assembled into an icosahedron with 20 facets and 12 vertices. The capsomeres forming the facets of the icosahedron are each surrounded by six other capsomeres and are called hexons, whereas the capsomeres forming the vertices are each surrounded by only five other capsomeres, and are called penton bases. Each penton base has a projection (fibre) attached. The combination penton base plus fibre is called a penton. In the complete virion 240 of the 252 capsomeres are hexons.

Harvests from tissue cultures contain soluble virus components as well as complete virus. These soluble components may consist of hexons, penton bases, fibres and pentons as well as polymeric forms of hexons, dimers and dodecomers of pentons (dodecons) and dimers of fibres. Hexons are the dominating soluble component.

TABLE 24  
QUANTITATION OF THE LATEX AGGLUTINATION TEST FOR  
STREPTOCOCCUS GROUP A WITH THE CELLOSCOPE SENSOR

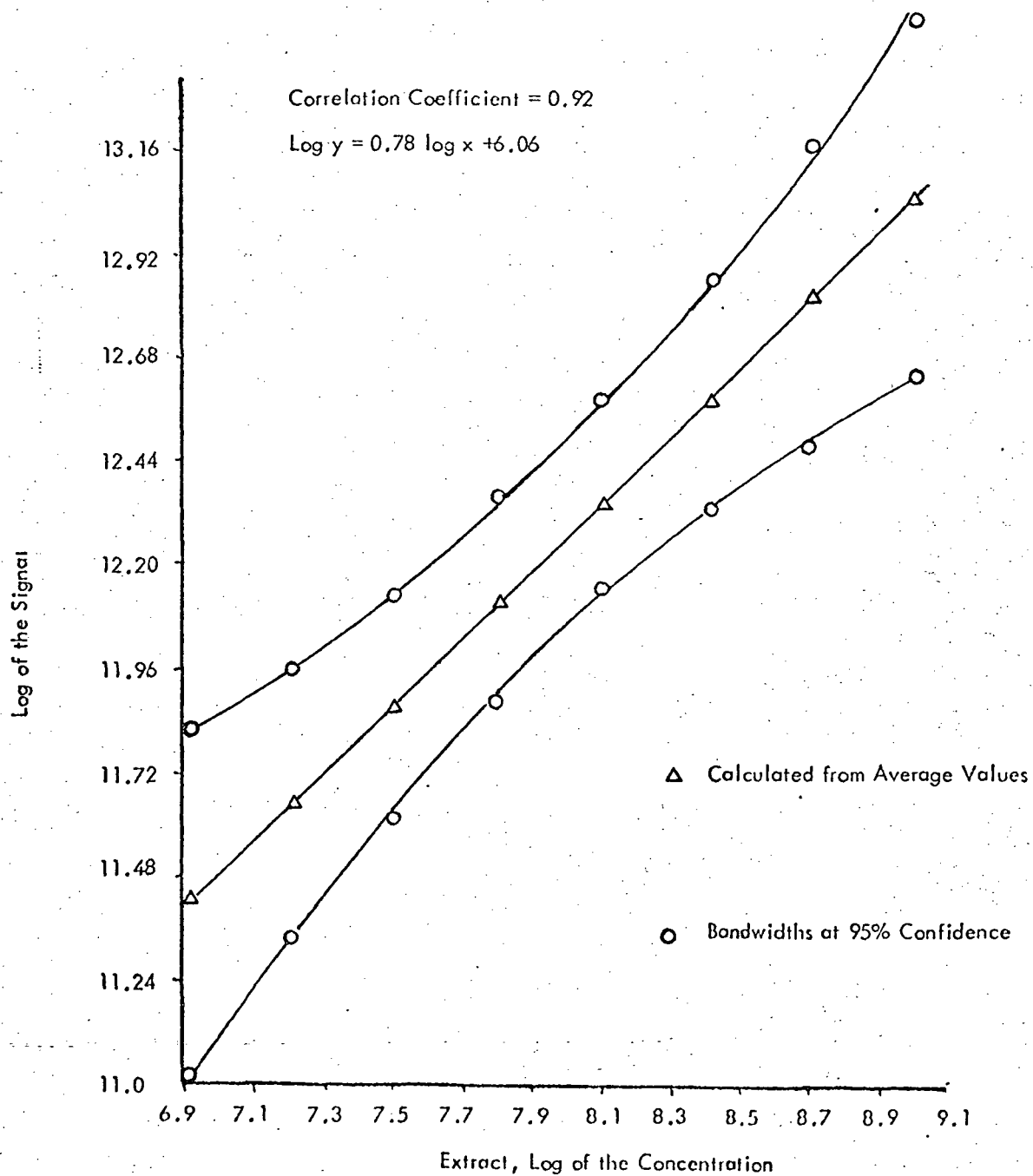
| Log<br>Concentration<br>(organisms/ml) | Celloscope Statistic |                      |                      |
|--|----------------------|----------------------|----------------------|
|  | Run #1<br>Log Signal | Run #2<br>Log Signal | Run #3<br>Log Signal |
| 9.0                                    | 12.99                | 12.79                | 12.29                |
| 8.7                                    | 12.98                |                      |                      |
| 8.4                                    | 12.89                | 12.43                | 12.25                |
| 8.1                                    | 12.67                |                      |                      |
| 7.8                                    | 12.45                | 12.31                | 11.80                |
| 7.5                                    | 12.18                |                      |                      |
| 7.2                                    | 11.63                | 11.40                | 11.44                |
| 6.9                                    |                      | 11.43                | 10.91                |

Correlation Coefficient = 0.915

Log y = 0.78 log x + 6.055

Standard Error of Estimate = 0.271

FIGURE 28  
QUANTITATION OF THE LATEX AGGLUTINATION TEST FOR  
STREPTOCOCCUS GROUP A WITH THE CELLOSCOPE SENSOR



The fibers are points of attachment for hemagglutination and give rise to antibodies that are type and inter-and intra-subgroup specific. The penton bases contain group, inter-and intra-subgroup specificities. The hexons provide group specificity as well as type specificity and inter-and intra-subgroup specificity as shown by complement fixation and immunoprecipitation.

The antigenic specificities of the hexon appear not to be uniformly distributed on its surface. The type specific antigen is available at the surface of the capsid, whereas the group specific antigen is localized at the inside of the capsid.

It thus appears that the only sources of group specific antigens are the hexons and the penton bases since the inter-subgroup specificities are limited in application. The great preponderance of hexons over the penton bases, both in the complete virus and in the unassembled soluble components, makes them the logical choice for the preparation of a group specific antigen.

#### 5.2.1 VIRUS PROPOGATION

Adenovirus types 1, 2, 3, 5 and 7 were graciously supplied by Dr. P. Byatt of the University of California at Los Angeles. Type 3 was selected as the candidate for isolation of the group antigen and the remaining types were frozen at -70 C for later use to determine if group antibody was produced.

The virus was propagated on HeLa cell cultures. The cells were grown either on surfaces of 32 oz. prescription bottles or in roller flasks using Eagle's Minimum Essential Medium with Hank's salts (HMEM) with 10% fetal bovine serum (FBS) and glutamine. Penicillin, streptomycin, kanamycin and amphotericin B were added to the medium to eliminate bacterial or fungal contamination. Monolayers were usually formed after 4-5 days incubation at 37 C. For infection of HeLa monolayers in 32 oz. prescription bottles, which were only used at the beginning of the program, the medium was poured off and the cells washed three times with Hank's Balanced Salt Solution (HBSS). Adenovirus type 3 in one ml of medium was introduced onto the monolayer and allowed to spread evenly. The bottles were returned to the incubator and the virus was allowed to adsorb to the cells for four hours. After the adsorption period, 40 ml of maintenance medium (HMEM with 2% FBS) was placed in the bottle and the cells

were incubated. Complete cytopathic effect occurred after 4-5 days. The cultures remained in the incubator for 2 days after complete CPE after which they were harvested.

HeLa cell growth in roller flasks were accomplished in a similar manner. The outgrowth medium was HMEM with 10% FBS and was discarded when a complete monolayer was formed. The cells were washed three times with HBSS and one ml of seed virus was placed in the bottle. Maintenance medium was placed in the roller bottle immediately and returned to the roller apparatus rotating at 0.5 rpm. Two days following complete CPE the bottles were harvested.

Harvesting prescription bottles was carried out by freezing at -70 C. The bottles were then thawed at room temperature until the ice was "slushy." At this time the ice was shaken over the glass surface to scrape any remaining cells off the glass. Cells adhering to roller flasks were scraped off with a special scraper. In either case the cells were frozen and thawed a total of three times, the cellular debris removed by low speed centrifugation and the virus titrated for HA and frozen at -70 C until used. HA titers of harvests from roller flasks ranged from 1:256 to 1:1024.

HA testing with adenovirus type 3 utilized African Green Monkey erythrocytes and .01 M phosphate buffered saline (PBS) as the diluent. Type 7 was also tested for HA in this manner. However, types 1, 2 and 5 display incomplete hemagglutination of rat erythrocytes, i.e., the individual virions attach to one RBC but for agglutination to occur a heterologous adenovirus anti-body must be present. For this reason, the PBS contained 1% of anti-adenovirus type 6 serum which had been absorbed with rat erythrocytes to remove any nonspecific hemagglutination.

Adenovirus types 1, 2, 5 and 7 were propagated in prescription bottles of HeLa cells. Type 7 had to be passed twice since the HA titer was low after the first pass. These heterologous types were propagated in order to confirm the group nature of antibodies produced. Table 25 gives the HA titers obtained from these viruses.



TABLE 25  
HEMAGGLUTINATION (HA) TITERS OF HARVESTS  
OF VARIOUS ADENOVIRUS TYPES

| Adenovirus | HA Titer |
|------------|----------|
| Type 1     | 1:128    |
| Type 2     | 1:32     |
| Type 5     | 1:64     |
| Type 7     | 1:64     |

#### 5.2.2 GROUP ANTIGEN PURIFICATION

Usually 1-2 liters of Adenovirus type 3 per batch was used to isolate and purify the group antigen. The crude virus harvest was concentrated by dialysis against sucrose to approximately 5% of its original volume and the residual sucrose was removed by dialysis against 0.02 M Tris buffer, pH 8.4. The concentrated crude virus was then centrifuged in the preparative ultracentrifuge at 37,000 x g for one hour to remove intact virions and other insoluble material. The soluble supernatant fluid was chromatographed on DEAE Sephadex A-25 in 0.02 M Tris buffer, pH 8.4 and eluted with a shallow NaCl gradient from 0 to 1.0 M in the tris buffer. Fractions, about 2 ml each, were monitored for U.V. absorbance at 280 nm, HA, and complement fixation (CF) activities. The fractions containing CF activity were concentrated then further purified by exclusion chromatography on G-200 Sephadex in Tris buffer. The fractions showing peak absorbance at 280 nm were titered for HA and CF. Those with maximum CF and minimum HA were combined and concentrated. This final material contained hexons and a small amount of pentons which demonstrate complete HA activity. This final G-200 purified material was used to immunize rabbits.

Table 26 shows a typical DEAE separation of the crude virus supernatant fluid. In this example fractions 85 to 100, containing most of the CF activity, were further purified on G-200 Sephadex. The CF activity in fractions 165 to 190 was probably due to a small amount of intact virions and was not included in further purification processes. This separation is graphically demonstrated in Figure 29.

TABLE 26

CF AND HA ACTIVITY OF FRACTIONS COLLECTED FROM  
THE CHROMATOGRAPHY OF BATCH #1 SOLUBLE  
ADENOVIRUS 3 COMPONENTS ON DEAE A-25 SEPHADEX

| Fraction # | CF Titer | HA Titer |
|------------|----------|----------|
| 5-75       | -        | -        |
| 80         | 10       | -        |
| 85         | 80       | -        |
| 90         | 160      | -        |
| 95         | 80       | -        |
| 100        | 20       | -        |
| 105        | 10       | 2,560    |
| 110        | 10       | 20,480   |
| 115        | 10       | 320      |
| 120        | -        | 320      |
| 125        | -        | 160      |
| 130        | -        | 160      |
| 135        | -        | 160      |
| 140        | -        | 160      |
| 145        | -        | 80       |
| 150        | -        | 80       |
| 155        | -        | 80       |
| 160        | -        | 80       |
| 165        | 10       | 80       |
| 170        | 20       | 160      |
| 175        | 20       | 320      |
| 180        | 20       | 640      |
| 185        | 20       | 160      |
| 190        | 20       | 160      |
| 195        | -        | 40       |
| 200-225    | -        | -        |

FIGURE 29

COMPLEMENT FIXATION (CF), HEMAGGLUTINATION (HA) AND ABSORBANCE AT 280 nm OF FRACTIONS ELUTED FROM THE CHROMATOGRAPHY OF SOLUBLE ADENOVIRUS TYPE 3 COMPONENTS ON DEAE SEPHADEX

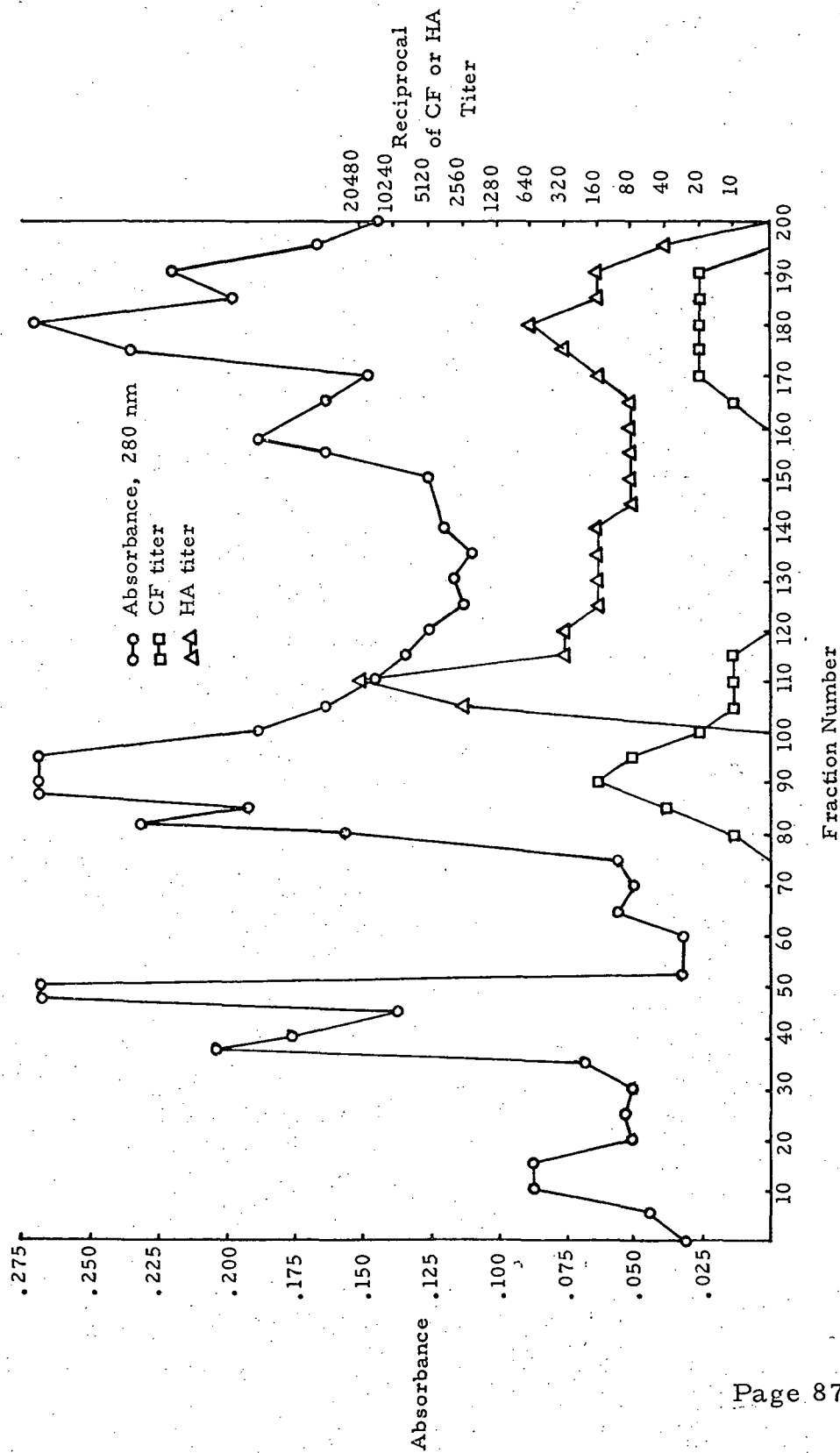


Table 27 shows CF and HA data obtained from the chromatography of the concentrated CF positive fractions of the aforementioned DEAE separation. Peak absorbance at 280 nm was between fractions 20 and 55. As can be seen CF activity was found in fractions 25 through 40 which were combined and concentrated for use.

TABLE 27  
HA AND CF ACTIVITY OF FRACTIONS COLLECTED  
FROM THE CHROMATOGRAPHY OF BATCH #1 DEAE  
PURIFIED MATERIAL ON G-200 SEPHADEX

| Fraction # | CF | HA |
|------------|----|----|
| 20         | -- | 4  |
| 25         | 8  | 8  |
| 30         | 16 | 8  |
| 35         | 32 | -- |
| 40         | 8  | -- |
| 45         | -- | -- |
| 50         | -- | -- |
| 55         | -- | -- |

Table 28 gives the data for all of the adenovirus group antigen batches that were produced.

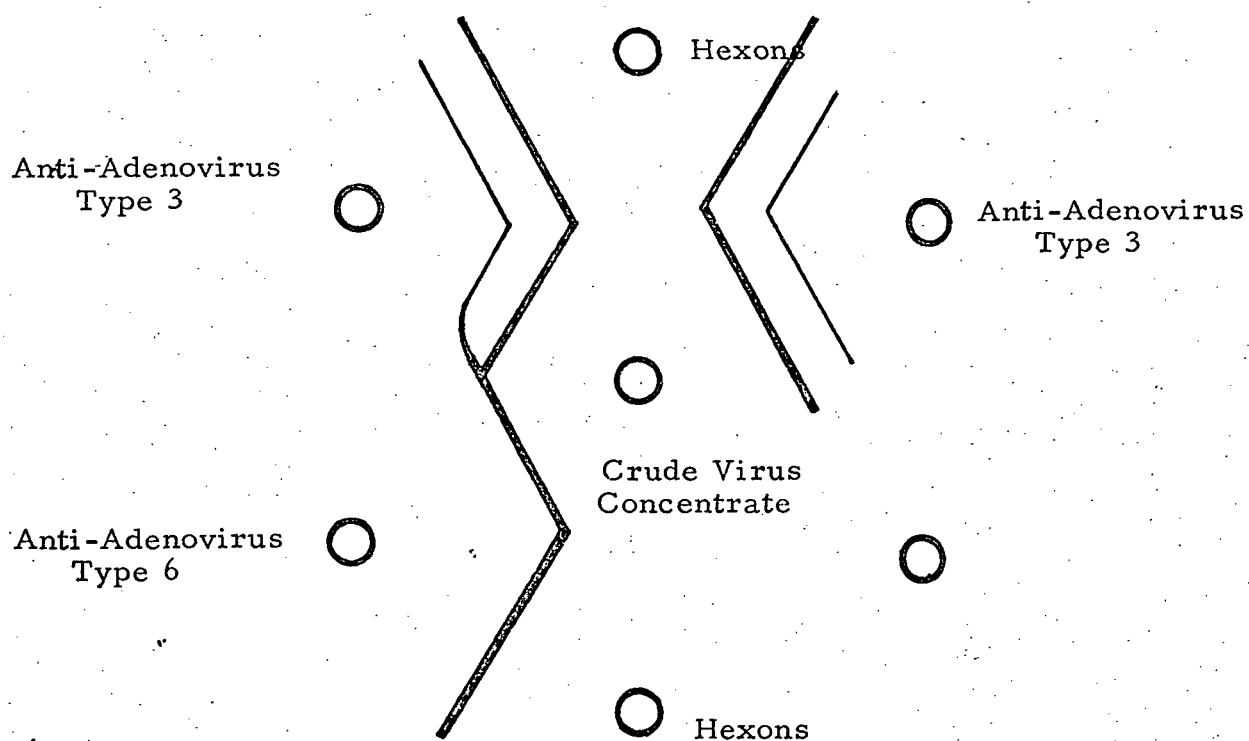
Figure 30 shows a diagram of a typical immunodiffusion plate and demonstrates two precipitin lines between a hexon preparation or concentrated crude virus and the commercial homologous antiserum while only one line appeared against a heterologous serum (anti-adenovirus type 6). The second line against homologous antisera was most likely due to a small amount of type specific antigen, probably pentons, which eluted along with the hexons following column chromatography. Batch #4 hexons, however, gave only one precipitin line when tested against homologous antisera. This was the only batch that did this and was obviously more pure than the others.

TABLE 28  
DATA FOR ADENOVIRUS GROUP ANTIGEN  
(HEXON) PRODUCTION

| Hexon<br>Batch # | Original<br>Crude Virus |          | Concentrated<br>Crude Virus<br>Supernate |          | Concentrated<br>Hexons |          |
|------------------|-------------------------|----------|--|----------|------------------------|----------|
|                  | Volume                  | HA titer | Volume                                   | HA titer | Volume                 | CF titer |
| 1                | 820 ml                  | 1:256    | 20 ml                                    | 1:10,240 | 9.2 ml                 | 1:64     |
| 2                | 1240 ml                 | 1:512    | 51 ml                                    | 1:20,480 | 8.0 ml                 | 1:512    |
| 3                | 1792 ml                 | 1:1280   | 55 ml                                    | 1:20,480 | 13.0 ml                | 1:640    |
| 4                | 1300 ml                 | 1:512    | 50 ml                                    | 1:20,480 | 15.5 ml                | 1:256    |
| 5                | 2000 ml                 | 1:1024   | 70 ml                                    | 1:20,480 | 15.0 ml                | 1:1024   |

FIGURE 30

RADIAL IMMUNODIFFUSION BETWEEN SOLUBLE ADENOVIRUS  
TYPE 3 COMPONENTS PURIFIED BY CHROMATOGRAPHY ON  
G-200 SEPHADEX AND ADENOVIRUS TYPES 3 AND 6 ANTISERA



### 5.2.3 GROUP ANTIBODY PRODUCTION

Antibody to the adenovirus group antigen was obtained by immunizing New Zealand white rabbits with the hexon preparations. The initial immunization schedule consisted of three weekly 1 ml intramuscular injections of the antigen emulsified with an equal volume of Freund's complete adjuvant (FAC) followed by a series of intravenous injections. The schedule is outlined in Table 29. Following the first bleeding the rabbits were maintained by the same series of weekly intravenous injections and were bled one month after the first bleeding then were exsanguinated one month after the second bleeding. A group of animals were similarly immunized with a control antigen consisting of medium from an uninfected HeLa cell culture.

TABLE 29  
SCHEDULE FOR THE IMMUNIZATION OF RABBITS WITH  
SOLUBLE ADENOVIRUS GROUP SPECIFIC ANTIGEN

| Day       | 1                 | 7                 | 14                | 21          | 22           | 23           | 35          | 36           | 37           | 42             |
|-----------|-------------------|-------------------|-------------------|-------------|--------------|--------------|-------------|--------------|--------------|----------------|
| Injection | 1 ml<br>IM<br>FCA | 1 ml<br>IM<br>FCA | 1 ml<br>IM<br>FCA | .5 ml<br>IV | 1.0 ml<br>IV | 2.0 ml<br>IV | .5 ml<br>IV | 1.0 ml<br>IV | 2.0 ml<br>IV | Trial<br>Bleed |

FCA = Freund's Complete Adjuvant

IM = Intramuscular

IV = Intravenous

After each bleeding CF titers were taken on sera obtained from each rabbit, the sera pooled, and CF titers were taken on the pool. The antigen in each case was 4 CF units (obtained by block titration against commercial type 3 antisera) of a hexon preparation. The CF titers for the adenovirus group antisera are given in Table 30. The CF titers of the antisera became progressively lower with each bleeding, however, the intensity of precipitin bands in immunodiffusion tests seemed to increase with each bleeding when tested against the same hexon preparation.

TABLE 30  
COMPLEMENT FIXATION TITERS OF ADENOVIRUS  
GROUP ANTISERA OBTAINED FROM RABBITS

| Antiserum   | 1st Bleeding<br>CF titer | 2nd Bleeding<br>CF titer | 3rd Bleeding<br>CF titer |
|-------------|--------------------------|--------------------------|--------------------------|
| Rabbit #1   | 1:64                     | 1:64                     | 1:32                     |
| Rabbit #2   | 1:128                    | 1:64                     | 1:32                     |
| Rabbit #3   | 1:32                     | 1:64                     | 1:32                     |
| Rabbit #4*  | 1:64                     | --                       | --                       |
| Rabbit #5   | 1:32                     | 1:64                     | 1:32                     |
| Pooled Sera | 1:128                    | 1:64                     | 1:32                     |

\* Rabbit #4 died after the first bleeding.

Immunodiffusion tests were performed after each bleeding against a hexon preparation and control antigen. Figure 31 shows a diagram of a typical test. The tests showed one line in common with both the group antigen and the control antigen which was either from medium components or the host cells and two precipitin lines against the group antigen only. The lines closest to the antisera wells were much heavier and would indicate the major virus antigen in the preparation. This probably represents the hexons. Other gel diffusion studies (not shown) testing a commercial human adenovirus reference serum against the group antigen also showed two precipitin lines and the one closest to the antiserum well was much heavier. These lines were certainly viral in nature.

There were other antibodies to the control antigen which did not appear in the tests against this particular batch of group antigen. The absence of precipitin lines in identity with these was probably due to reduced amounts of contaminating medium components in later batches of group antigen.



FIGURE 31  
RADIAL IMMUNODIFFUSION OF RABBIT ANTISERA

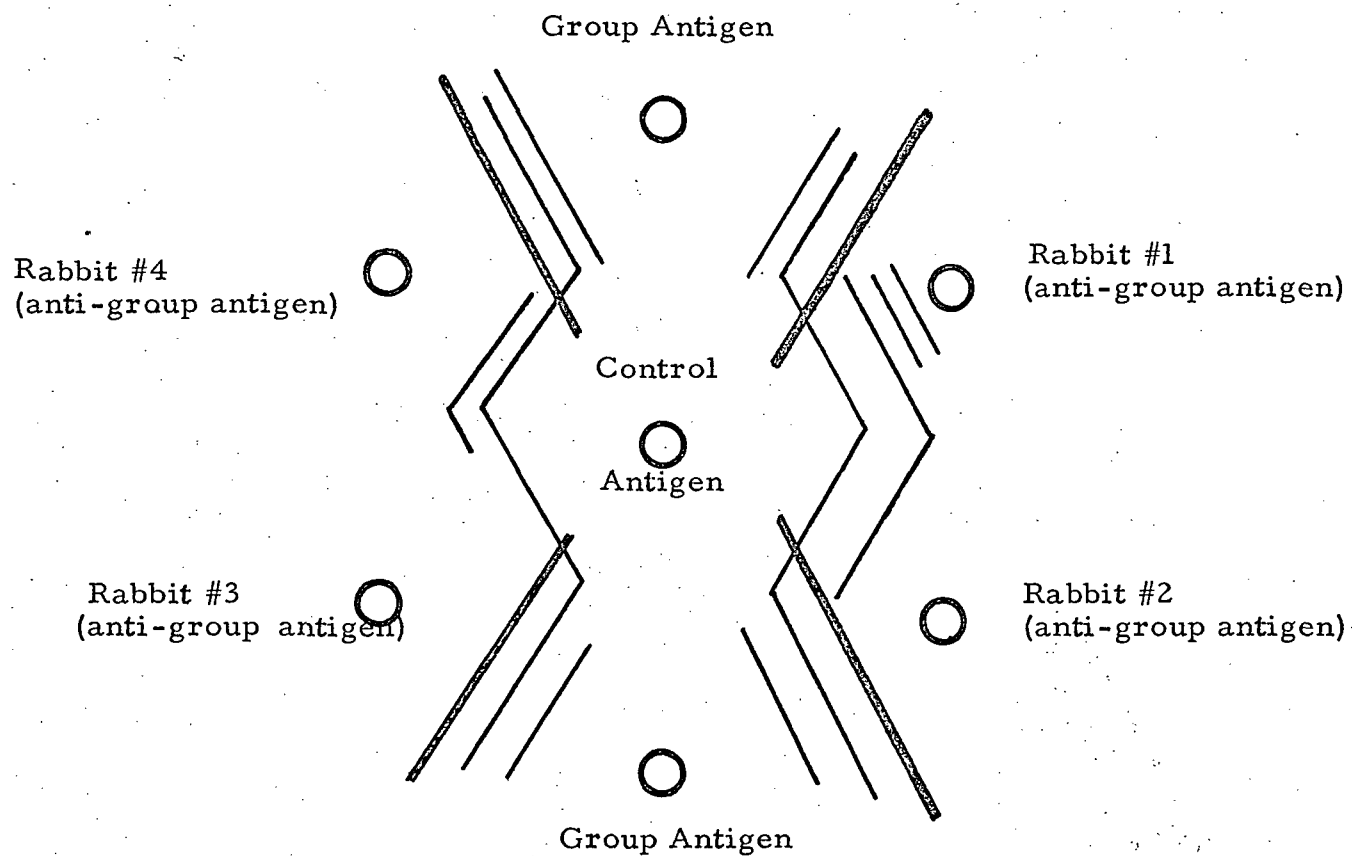


Figure 32 are the diagrams of immunoelectrophoresis plates which also demonstrate that antibodies were produced to medium components. In this test the hexons were subjected to electrophoresis and then allowed to react with the group antibody, antibody to the control antigen or the commercial adenovirus reference serum. As can be seen precipitin lines developed and the group antibody showed two arcs not in common with the control antiserum. The human reference serum also showed two arcs, neither of which was in common with the control antiserum. Two precipitin lines were formed against the control antibody which did not appear with the adenovirus group antibody which would indicate that these impurities were absent or not at a high enough level in the group antigen to illicit an immune response in the rabbits.

Later tests by immunoelectrophoresis showed the contaminating antibodies to be against the fetal bovine serum in the cell culture medium. Block precipitation titrations were performed using two-fold dilutions of group antibody and fetal bovine serum. These tests showed the optimum proportions necessary to obtain maximum precipitation. Oddly enough, the tests were identical for sera from all three bleedings. To absorb the antibodies to fetal bovine serum from the sera one volume of antiserum was mixed with an equal volume of fetal bovine serum diluted 1:2 with PBS. The mixture was incubated one hour at 37 C, then overnight at 4 C. The precipitate was removed by centrifugation and tested by CF, immunodiffusion and immunoelectrophoresis. The CF titers dropped, as would be expected, to one-half the original titer.

A diagram of an immunodiffusion test is given in Figure 33. The untreated antiserum gave precipitin lines against both fetal bovine serum and the control antigen. The absorbed antiserum did not give these lines. Both sera produced lines against the hexon preparation.

The immunoelectrophoresis tests that were performed (Figure 34) confirm the presence of antibodies to the group antigen but not to host cell or medium components. In Figure 34a, hexons were tested against both the human reference serum and the absorbed antibody and showed identical lines. Since the human serum had no antibodies to cell culture components, the lines were viral in nature. In Figures 34b, 34c, and 34d, both untreated antiserum

FIGURE 32

IMMUNOELECTROPHORESIS OF RABBIT ANTISERA AND  
HUMAN ADENOVIRUS REFERENCE SERA

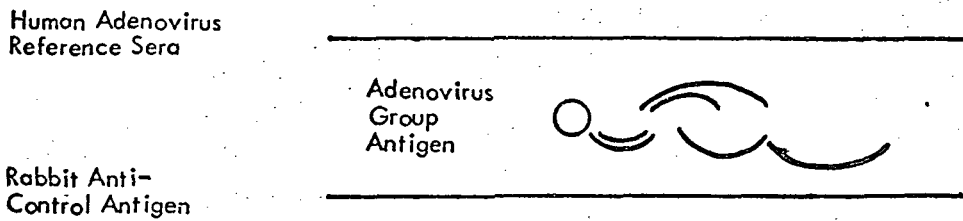
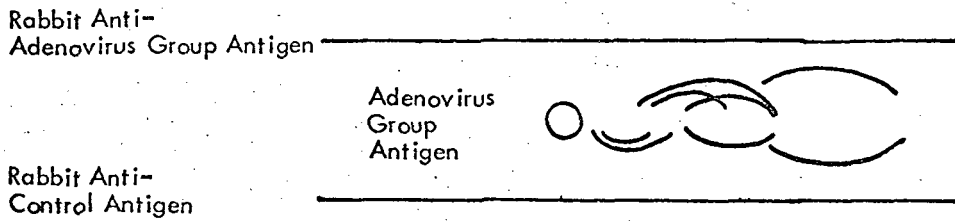


FIGURE 33  
GEL DIFFUSION OF ADENOVIRUS GROUP ANTISERUM  
UNTREATED AND ABSORBED WITH FETAL BOVINE SERUM

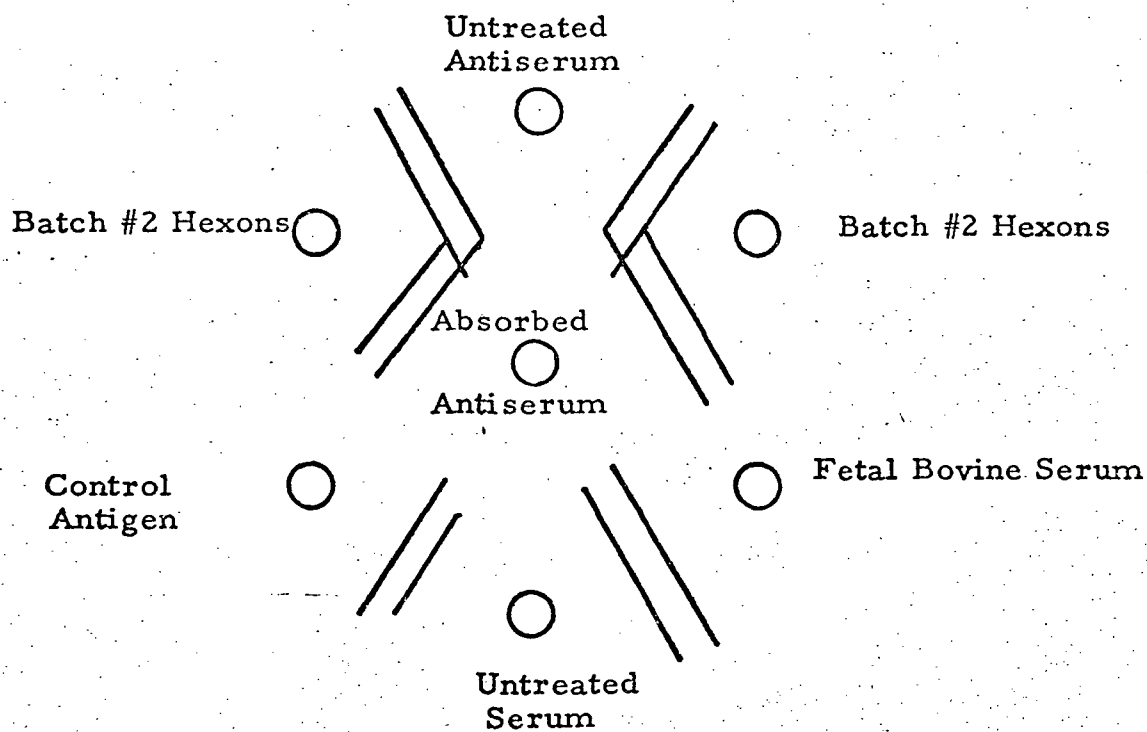
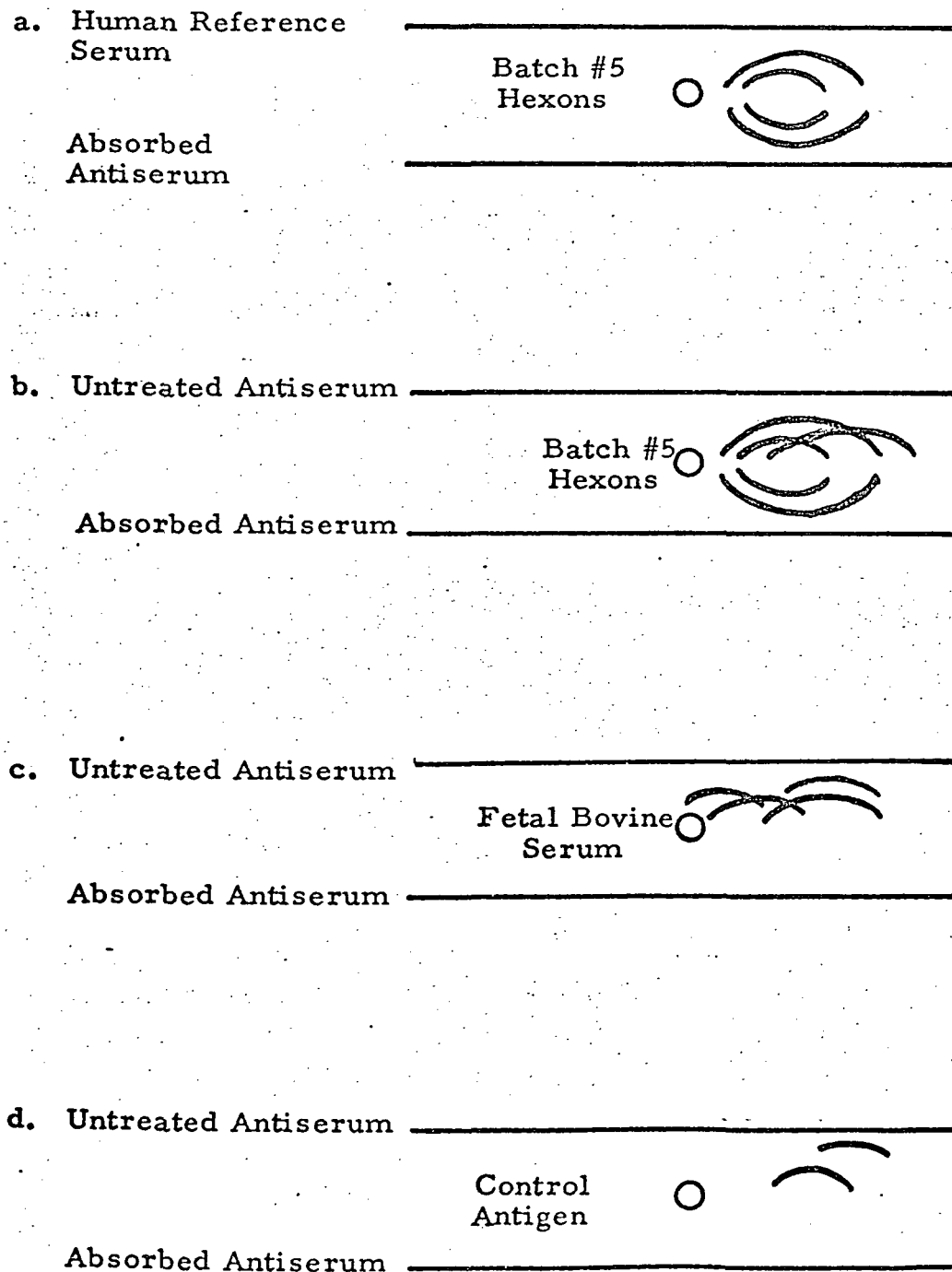


FIGURE 34  
IMMUNOELECTROPHORESIS OF PURIFIED  
ADENOVIRUS GROUP ANTIBODY



and absorbed antiserum were tested against hexons, fetal bovine serum and control antigen respectively. The test with the hexon preparation showed an extra line against the untreated antiserum which was not present when tested against the absorbed antiserum. The last two tests show the absence of lines in the absorbed antibody against either fetal bovine serum or the control antigen.

The reason for absorbing out the antibodies to fetal bovine serum was because all testing was against adenoviruses which had been propagated in media containing fetal bovine serum. For detection of adenovirus in clinical samples the use of antibody which had been absorbed would not be necessary.

The heterologous adenovirus types (1, 2, 5 and 7) were tested for CF using absorbed antisera from the third bleeding. Four units of antibody were used. The results of these tests are given in Table 31. As can be seen the control, medium from a HeLa cell culture grown at the same time as the viruses, showed no CF activity while the virus cultures did.

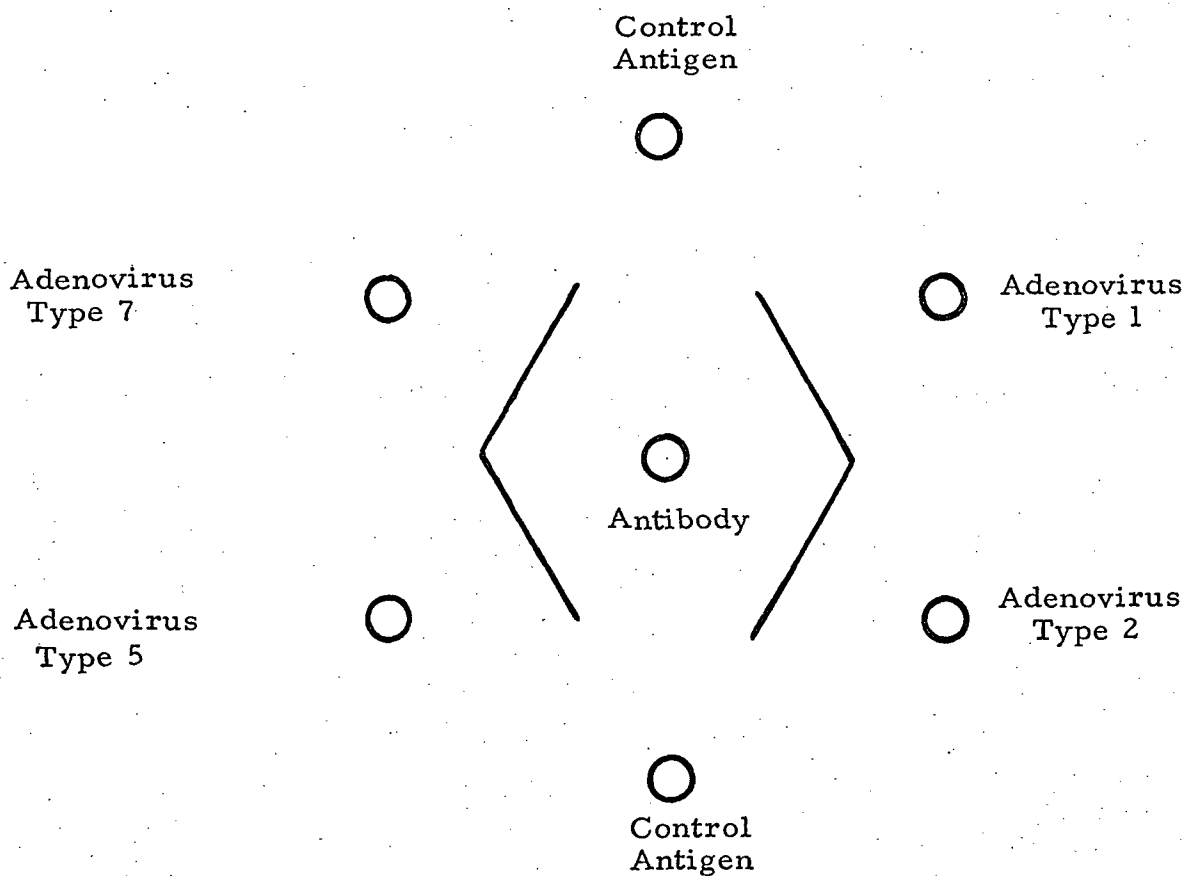
Immunodiffusion tests were also performed and are diagrammed in Figure 35. As can be seen precipitin lines were formed only where the adenovirus was present and not with the control antigen.

From the data presented it is obvious that antibody to the adenovirus group antigen was produced and any antibodies to medium components could be removed.

TABLE 31  
COMPLEMENT FIXATION TITERS OF VARIOUS  
ADENOVIRUS TYPES USING FETAL BOVINE  
ABSORBED ADENOVIRUS GROUP ANTIBODY

| Adenoviruses | CF titer |
|--------------|----------|
| Type 1       | 1:16     |
| Type 2       | 1:16     |
| Type 5       | 1:16+    |
| Type 7       | 1:16     |
| Control      | Negative |

FIGURE 35  
IMMUNODIFFUSION OF VARIOUS ADENOVIRUS TYPES  
USING FETAL BOVINE SERUM ABSORBED ADENOVIRUS  
GROUP ANTIBODY



#### 5.2.4 IMMUNOGLOBULIN PURIFICATION AND BEAD SENSITIZATION

The immunoglobulins from FBS absorbed adenovirus group antibody were isolated by two precipitations with 50% saturated ammonium sulfate. The final precipitate was redissolved in a volume of .02 M phosphate buffer, pH 7.6 equal to one half the original serum volume. The immunoglobulin solution was then dialyzed against the same buffer to remove the ammonium sulfate. The immunoglobulin was purified by chromatography on a mixed bed DEAE-G50 Sephadex column, filtered through a 0.22  $\mu$  Millipore filter then measured for protein content by U. V. absorbance at 276.

Beads (1.1  $\mu$ ) were sensitized as described earlier using 5 mg protein per ml of stock bead suspension. Sensitization took place in glycine buffered saline at room temperature for one hour.

#### 5.2.5 DETECTION OF ADENOVIRUS BY PIA

Because of time limitations, optimization of conditions for PIA reactivity using adenovirus group antibody sensitized beads, could not be completed. The reaction conditions used were those that were determined as optimum for the detection of influenza. The main testing was performed with the celloscope conductrometric system using 1.1  $\mu$  sensitized beads under conditions described previously for the influenza system.

The first series of PIA testing involved the detection of dilutions of a hexon preparation. The hexons, having a CF titer of 1:640, were first diluted 1:100 in GBS then two-fold dilutions were made to 1:51,200. Equal volumes (0.5 ml) of hexon dilution and sensitized beads (washed and diluted 1:250) were mixed and incubated for one hour at 37 C. After incubation 0.15 ml of reacted beads were added to 50 ml of twice filtered (0.22  $\mu$ ) 1% NaCl. Agglutination was detected with the Celloscope and the results are given in Table 32 and graphically shown in Figure 36. When the results are compared to the S/N ratio obtained by comparing duplicate controls it appears that the system detected the hexons at a 1:3200 dilution. Higher dilutions gave S/N values slightly higher than the controls, although for this study this was considered in the noise area. This dilution of hexons would be equal to 0.2 CF units if one defines one CF unit as equal to the highest dilution of hexons that will demonstrate complete fixation of complement. This batch of hexons, undiluted, would then contain 640 CF units per test volume (.025 ml).



TABLE 32  
DETECTION OF ADENOVIRUS GROUP ANTIGEN  
(HEXONS) BY USE OF THE CELLOSCOPE

| Hexon Dilution | Celloscope Signal     | S/N  |
|----------------|-----------------------|------|
| 1:100          | $5.13 \times 10^{10}$ | 2.87 |
| 1:200          | $3.49 \times 10^{10}$ | 1.95 |
| 1:400          | $2.55 \times 10^{10}$ | 1.42 |
| 1:800          | $3.09 \times 10^{10}$ | 1.73 |
| 1:1600         | $2.20 \times 10^{10}$ | 1.23 |
| 1:3200         | $2.72 \times 10^{10}$ | 1.52 |
| 1:6400         | $2.31 \times 10^{10}$ | 1.29 |
| 1:12800        | $1.95 \times 10^{10}$ | 1.09 |
| 1:25600        | $2.20 \times 10^{10}$ | 1.23 |
| 1:51200        | $1.98 \times 10^{10}$ | 1.11 |
| Control #1     | $1.79 \times 10^{10}$ | --   |
| Negative       | $1.73 \times 10^{10}$ | 0.97 |

The ability of beads sensitized with adenovirus group antibody was tested under the conditions previously noted against heterologous adenovirus types, 1, 2, 5 and 7. The viruses were HeLa cell culture harvests in the culture medium (HMEM, 2% fetal bovine serum). The control in this test was medium from an uninfected HeLa cell culture. Duplicate controls were tested and compared to produce a baseline S/N value. The results of the test are given in Table 33. As can be seen S/N ratios were higher for the heterologous adenovirus types when compared to the control S/N. These adenovirus harvests were not treated in any way to release hexons. Such treatment might have produced higher S/N ratios.

FIGURE 36  
DETECTION OF ADENOVIRUS GROUP ANTIGEN  
(HEXONS) BY USE OF THE CELLOSCOPE

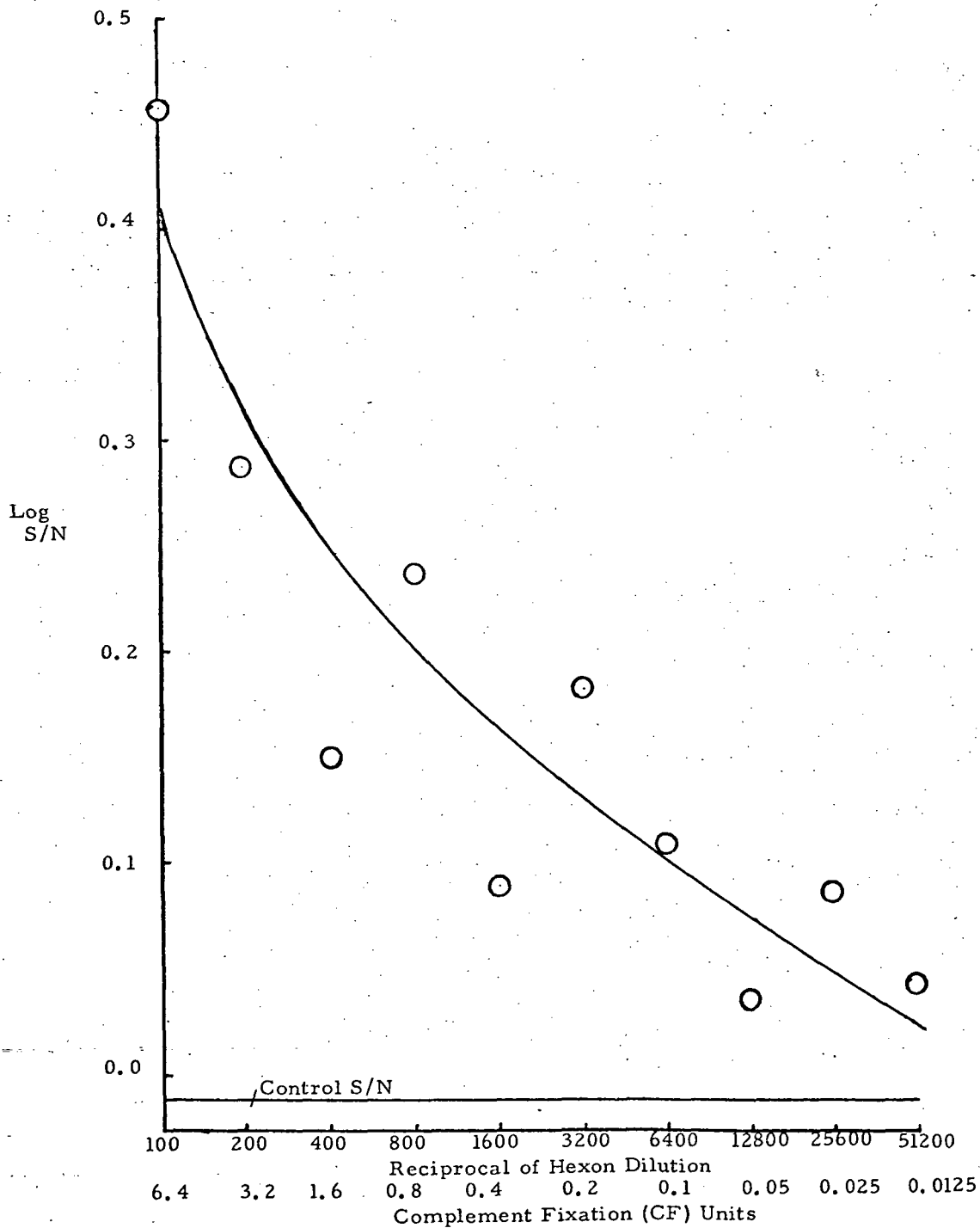


TABLE 33

DETECTION OF ADENOVIRUS TYPES 1, 2, 5 AND 7  
WITH BEADS SENSITIZED WITH ADENOVIRUS GROUP  
ANTIBODY PRODUCED IN RABBITS IMMUNIZED WITH  
ADENOVIRUS TYPE 3 HEXONS

| Test Sample       | Signal                | S/N  |
|-------------------|-----------------------|------|
| Adenovirus Type 1 | $2.89 \times 10^{10}$ | 1.56 |
| Adenovirus Type 2 | $3.92 \times 10^{10}$ | 2.12 |
| Adenovirus Type 5 | $3.37 \times 10^{10}$ | 1.82 |
| Adenovirus Type 7 | $3.87 \times 10^{10}$ | 3.09 |
| Negative          | $2.13 \times 10^{10}$ | 1.15 |
| Control           | $1.85 \times 10^{10}$ | --   |

#### 5.2.5.1 Unknown Clinical Samples

A series of unknown virus samples were supplied by NASA to further test the ability of this bead system to detect adenoviruses. Seven of the unknown samples were supplied in Eagle's Basal Medium with Earle's salts (EBME) and 2% fetal bovine serum, and three were in Earle's salts with 0.5% gelatin. Solutions were prepared of each of these media and each sample was compared with its own type of control. As in the previous tests two separate controls were tested against each other to form a baseline S/N value. The unknown samples were also tested for CF by using fetal bovine serum-absorbed adenovirus group antibody prepared on this program. These results are given in Table 34. One immediately notes the enormous S/N ratio given by sample #16. The CF titer (1:4) would not indicate that there was sufficient antigen to give such a high S/N. This particular sample, although said to be in EBME with 2% fetal calf serum, appeared much different than the rest of the samples in the same medium. It was darker in color and had a heavy precipitate. The PIA results for this sample are probably in error because controls in the PIA test must be as close as possible in composition to the test specimen at the present state of the test system. Of the remaining samples supplied in EBME with 2% fetal calf serum sample 2 was definitely positive by PIA but there was insufficient sample to perform the CF test. Samples 4, 6 and 9 were also definitely positive. Unknown 12 although having a 1:8 titer in the CF test did not give a sufficient S/N value to be considered positive by PIA. No explanation can be given for this. Unknown 14 was definitely negative as there was no complement fixation by the adenovirus group antibody and the S/N value in the PIA test was lower than the control negative.

The three samples supplied in Earle's salts with 0.5% gelatin showed S/N values of from 3 to 4 times that of the control negative. Samples 5 and 11, however, displayed marginal fixation of complement while sample 1 was definitely positive by CF.

TABLE 34

DETECTION OF ADENOVIRUS IN UNKNOWN SAMPLES BY  
PASSIVE IMMUNE AGGLUTINATION AND COMPLEMENT  
FIXATION USING ADENOVIRUS GROUP ANTIBODY

| Sample      | CF titer | Celloscope Signal    | S/N     | (+) or (-) for Adenovirus from PIA Results |
|-------------|----------|----------------------|---------|--|
| Unknown #2  | *        | $1.1 \times 10^{11}$ | 23.40   | +  |
| Unknown #4  | 1:4      | $1.1 \times 10^{10}$ | 2.34    | +  |
| Unknown #6  | 1:8      | $2.4 \times 10^{10}$ | 5.11    | +  |
| Unknown #9  | 1:8      | $5.7 \times 10^{10}$ | 12.10   | +  |
| Unknown #12 | 1:8      | $5.1 \times 10^9$    | 1.09    | -  |
| Unknown #14 | Negative | $6.4 \times 10^9$    | 1.36    | -  |
| Unknown #16 | 1:4      | $5.5 \times 10^{12}$ | 1170.00 | +  |
| Negative A  | Negative | $6.7 \times 10^9$    | 1.43    |  |
| Control A   | --       | $4.7 \times 10^9$    |         |  |
| Unknown #1  | 1:32     | $1.9 \times 10^{10}$ | 4.32    | +  |
| Unknown #5  | weak 1:2 | $1.3 \times 10^{10}$ | 2.95    | +  |
| Unknown #11 | weak 1:2 | $1.5 \times 10^{10}$ | 3.40    | +  |
| Negative B  | Negative | $4.2 \times 10^9$    | 0.95    |  |
| Control B   | --       | $4.4 \times 10^9$    |         |  |

Negative A and Control A = Eagle's Basal Medium, Earle's Salts + 2% fetal calf serum.

Negative B and Control B = Earle's Salts + 0.5% gelatin.

(\*) Insufficient sample to perform CF test.

In order to obtain valid results when using the PIA system to detect microorganisms in clinical samples, three items must be taken into consideration.

1. The procurement of the sample must be performed in a manner that is conducive to the space environment.
2. The method of sample procurement should be one that will allow for maximum collection of the microorganism.
3. Treatment of the sample after collection must eliminate or markedly reduce any interference from the fluids or tissues from which the sample was obtained.

Ultimate solution of the first item must be obtained by trials in space. However, in an attempt to demonstrate the feasibility of some collection methods the following experiment was performed.

Three methods of sampling from the oropharynx were performed by two individuals who are known carriers of Mycoplasma salivarium, a common inhabitant of the saliva and oropharynx of normal humans. The three methods were:

1. Washing the mouth while attempting to gargle with the face down to simulate zero gravity. Twenty ml of phosphate buffered saline (PBS) was washed in the mouth for one minute in this manner.
2. Swabbing the throat of the two individuals and rinsing the swabs by shaking vigorously in five ml of PBS for one minute. Three such rinses were made and samples taken from each rinse.
3. Taking a mouth wash as in the first method. After one minute the PBS was returned to a flask. Three "inhalatory snorts" were then performed and after each, the material was placed in the same flask. The "inhalatory snort" consists of vigorously drawing back the nasal contents into the throat, then clearing the throat and emptying the specimen into the flask.

One of these methods was performed on three consecutive days. After the samples were collected, serial ten-fold dilutions were made in PBS and 0.1 ml of the dilution was plated onto replicate PPLO agar plates which contained penicillin and thallium acetate to eliminate bacterial contamination. PPLO agar is a common medium for the isolation and cultivation of mycoplasmas. The plates were incubated at 37 C for 5 to 7 days in an atmosphere of 95% N<sub>2</sub> and 5% CO<sub>2</sub>. After incubation the colonies were counted and the results expressed in colony forming units per ml of sample (CFU/ml). The results are shown in Table 35.

The results show that both methods of mouth wash were far superior to the throat swab for collecting microorganisms from the oropharynx under the conditions of the test. In washing the mouth with the face down it was almost impossible for the collecting fluid to reach the back of the throat without swallowing it. Because of this, the mouth wash alone probably did not allow collection of organisms from the throat. The "inhalatory snort" allowed about twice the number of mycoplasmas to be recovered from both individuals but if the fluid could have been gargled vigorously the recovery may have been much higher. More vigorous swabbing may have resulted in more organisms recovered in that method but toxicity of the swab must also be taken into account.

TABLE 35.

RECOVERY OF MYCOPLASMA SALIVARIUM  
FROM THE OROPHARYNX OF NORMAL HUMANS  
BY VARIOUS METHODS

| Method of collection    | Recovery (CFU/ml)  |                    |
|-------------------------|--------------------|--------------------|
|                         | Subject 1          | Subject 2          |
| 1. Mouthwash            | 2. $4 \times 10^2$ | 3. $5 \times 10^3$ |
| 2. Swab                 |                    |                    |
| 1st Rinse               | None               | 6. $3 \times 10^2$ |
| 2nd Rinse               | $0.5 \times 10^1$  | 2. $5 \times 10^1$ |
| 3rd Rinse               | none               | $0.5 \times 10^1$  |
| 3. Mouthwash            | $6.5 \times 10^2$  | $9.2 \times 10^3$  |
| with "inhalatory snort" |                    |                    |



Two commercially available sputum digestants were tested for their ability to reduce any interference saliva might have on the PIA test. The sputum digestants were Sputolysin (Calbiochem) and TB-Gest (J. T. Baker). In this test purified Adenovirus type 3 was used to represent a positive sample and the results were obtained by use of the Celloscope.

The experiment was performed in the following manner.

1. A test subject washed his mouth and gargled with 20 ml of GBS for one minute and returned the fluid to a flask.

2. Three "inhalatory snorts" were performed and the material from each was placed in the same flask. This material was used for all the tests.

3. For an untreated control series 0.1 ml Adenovirus type 3 in 0.02 M Tris, pH 8.4 was added to 0.9 ml of the wash fluid. Negative controls consisted of 0.9 ml of either GBS or wash fluid to which 0.1 ml of Tris was added. A positive control was made by diluting the purified adenovirus 1:10 in GBS. For this series the wash fluid was centrifuged at low speed to remove particulate material.

4. To one volume of uncentrifuged wash fluid an equal volume of Sputolysin (dithiothreitol) was added. The tube was mixed on a mechanical mixer for 30 seconds and the mixture allowed to stand at room temperature for 15 minutes. The tube was then centrifuged at low speed for 5 minutes. To 0.9 ml aliquots of the digested wash fluid either 0.1 ml of purified adenovirus or 0.1 ml Tris (negative wash control) was added. As a GBS control equal volumes of Sputolysin and GBS were treated in the same manner as the wash.

5. For tests using TB-Gest (N-acetyl-L-(-)-cysteine) a 0.05M solution was prepared in GBS. To 1.6 ml of wash fluid 0.4 ml of TB-Gest solution was added. The mixture was incubated at 37° C for 30 minutes then centrifuged. To 0.9 ml aliquots, 0.10 ml of either adenovirus or Tris was added. A GBS control was also prepared by treating GBS with TB-Gest then adding Tris buffer.

6. All samples were tested by reacting them with  $1.1\mu$  beads sensitized with adenovirus group antibody and agglutination was detected with the celloscope system.

The results are given in Table 36.

When one compares untreated GBS (GBS control) with the treated or untreated wash fluids there was a slight increase in signal to noise ratios (S/N) in each case. The sample treated with cysteine seemed to give the lowest S/N when compared to the GBS control (S/N = 1.68). Both untreated and Sputolysin treated wash fluids gave similar S/N values when compared to this control. When the wash fluid in each series was compared to its own GBS control both treated samples showed lower S/N ratios than untreated samples and the cysteine treated sample actually gave a fractional result. In this series of experiments the problem appeared to be not excess agglutination but one of diminishing signal in the samples which contained adenovirus. The adenovirus positive control in GBS gave a signal about 100 times those in any of the three test series. Also, if one compares each adenovirus positive test with its own negative wash fluid all gave similar S/N ratios and all were much lower than the GBS control series.

Apparently some substance in saliva or throat washings either broke up the clumps or diminished sensitized bead reactivity in some manner and was not eliminated by the chemical treatments tested. Time prevented further pursuit of this problem which must be resolved if the PIA is to be used for microbial detection.

In an effort to increase the amount of group antigen available for reaction with group antibody, Adenovirus type 3 virions which had been purified by density gradient centrifugation were treated by various methods of disruption. One ml aliquots of the virions were treated with 0.02% or 0.2% sodium lauryl sulfate (final concentration) at room temperature for one hour. After this treatment the samples were dialyzed against Tris buffer overnight. In another test, the virions were heated at 52 C for 10 minutes and 30 minutes. Both sodium lauryl sulfate and heat treatment

TABLE 36

EFFECT OF TREATMENT OF OROPHARYNGEAL WASHES WITH SPUTUM  
DIGESTANTS ON REACTIVITY OF ADENOVIRUS GROUP ANTIBODY  
SENSITIZED BEADS

| Sample                                     | Signal                | S/N <sup>1</sup> | S/N <sup>2</sup> | S/N <sup>3</sup> |
|--|-----------------------|------------------|------------------|------------------|
| GBS Control                                | $3.04 \times 10^9$    |                  |                  |                  |
| GBS + Adenovirus                           | $4.64 \times 10^{11}$ | 153.00           |                  | 153.00           |
| Untreated Wash fluid                       | $7.59 \times 10^9$    | 2.50             |                  | 2.50             |
| Untreated Wash fluid + Adenovirus          | $4.29 \times 10^{10}$ | 14.10            | 5.65             | 14.10            |
| Sputolysin treated GBS Control             | $4.03 \times 10^9$    |                  |                  | 1.33             |
| Sputolysin treated Wash fluid              | $7.93 \times 10^9$    | 1.97             |                  | 2.61             |
| Sputolysin treated Wash fluid + Adenovirus | $7.77 \times 10^{10}$ | 19.30            | 9.80             | 25.6             |
| TB-Gest treated GBS Control                | $5.8 \times 10^9$     |                  |                  | 1.91             |
| TB-Gest treated Wash fluid                 | $5.11 \times 10^9$    | 0.88             |                  | 1.68             |
| TB-Gest treated Wash fluid + Adenovirus    | $3.82 \times 10^{10}$ | 6.57             | 7.48             | 12.60            |

N<sup>1</sup> = GBS control in each series

N<sup>2</sup> = Wash fluid in each series

N<sup>3</sup> = GBS control

have been described as methods for adenovirus disruption. CF and HA tests were performed and the results are given in Table 37. For the CF test, the adenovirus group antibody was used. As can be seen, the HA titers were lowered in all cases. This may have been caused by disintegration of the hemagglutinating structures (fibers) on the virions. Treatment with 0.02% sodium lauryl sulfate seemed to give the highest CF titer. The more concentrated sodium lauryl sulfate treatment may have destroyed some of the CF antigen as this should have produced more disruption. Heat treatment did not result in as high CF titers.

The samples that were treated with sodium lauryl sulfate were then tested for PIA reactivity in the celloscope. The results are given in Table 38. As with the CF tests treatment with 0.02% sodium lauryl sulfate gave a better reaction with the group antibody than did 0.2%. However, the untreated sample gave a much higher signal. Apparently the group antibody contains antibodies other than the CF antibody. These antibodies are apparently directed toward antigenic determinants which are unstable in the presence of sodium lauryl sulfate. If this were the case, perhaps the benefit derived by releasing hexons was overwhelmed in untreated homologous virions by the reaction of the pentons with the specific antibodies. The test should be repeated using heterologous adenoviruses in order to determine whether or not such treatment increases the signal in the PIA test.

TABLE 37

THE EFFECT OF VARIOUS METHODS OF DISRUPTION OF  
ADENOVIRUS TYPE 3 VIRIONS ON CF AND HA TITERS

| Treatment                   | HA titer | CF titer |
|-----------------------------|----------|----------|
| Untreated Control           | 1:2048   | 1:32     |
| 0.02% Sodium Lauryl Sulfate | 1:512    | 1:128    |
| 0.2% Sodium Lauryl Sulfate  | 1:64     | 1:64     |
| 52C, 10 minutes             | 1:256    | 1:64     |
| 52C, 30 minutes             | 1:256    | 1:32     |

TABLE 38

THE EFFECT OF DISRUPTION OF ADENOVIRUS TYPE 3  
VIRIONS WITH SODIUM LAURYL SULFATE ON THE PIA  
REACTION

| Sample                  | Signal               | S/N    |
|-------------------------|----------------------|--------|
| Untreated Control       | $5.3 \times 10^{11}$ | 123.30 |
| Treated with 0.02% SLS* | $1.2 \times 10^{11}$ | 27.90  |
| Treated with 0.2% SLS   | $1.6 \times 10^{10}$ | 3.72   |
| Negative Control        | $4.6 \times 10^9$    | 1.07   |
| Noise Control           | $4.3 \times 10^9$    | --     |

\*SLS = Sodium Lauryl Sulfate

## 6.0 CONCLUSIONS AND RECOMMENDATIONS

The PIA test for microbial detection has advanced from a totally manual test which was time consuming and cumbersome to one which at the present is fully automated except for sample preparation and is rapid and very simple to perform. Optimum conditions for the influenza and mycoplasma systems have been determined. The sensitivity of the test for detection of Influenza A<sub>2</sub>, Hong Kong is very good and the test should have little difficulty in detecting this virus in clinical samples. The sensitivity of the test to detect Mycoplasma pneumoniae was not as good as anticipated. This was probably caused by the nature of the mycoplasma antigen. Also, at the time this study was undertaken, the immunology of Mycoplasma pneumoniae was not well understood. Recent advances in this area including the use of "not cell-bound" antigens may be used to increase PIA sensitivities.

Several sensor systems were tested as candidates to automate the readout of the PIA reaction. Two systems were chosen, the celloscope which measures differences in size of bead clumps by conductivity changes and the photometric system which measures differences in light transmission between agglutinated and unagglutinated samples. Both instruments compared favorably to the manual PIA test and were much more rapid.

Based upon a breadboard design a photometric test bed device was fabricated, and tested.

The PIA reaction was tested for feasibility in detecting a bacterium, Group A streptococcus. Sensitivity was poor, but the antibody used was non-precipitating so maximum agglutination could not be expected to occur. When optimum reaction conditions and the proper type of antibody is used sensitivity should be at an acceptable level.

The feasibility of using group antigens for detecting viruses was demonstrated. The use of antibody to an antigen that is common to all members of a virus group would greatly reduce the number of tests necessary to determine the identity of the virus in question. In almost all cases

identification of the virus group would be all that is necessary for diagnosis of a particular viral disease. In the present study an antigen common to all adenoviruses of human importance was isolated and antibody to this antigen was produced. The antibody reacted with four heterologous adenovirus types by complement fixation, immunodiffusion and PIA. The sensitivity of the group antibody-sensitized beads to detect heterologous adenovirus types was not determined. This should be done and optimum conditions for the reaction should be determined as well.

The group antigen approach should be investigated for other viral groups.

Influenza offers an excellent example of a viral group where this could be applied. There are three distinct serologic types of influenza viruses; A, B and C. Types A and B are most commonly involved in human disease. These two types, especially type A, show further variations within types which are caused by changes in dominance of types of hemagglutinin or types of neuraminidase antigens. These antigens are continually changing so that immunization with a given influenza virus does not give total immunity to a new influenza virus.

Since antisera produced to a given influenza virus may not react optimally to another virus of the same type, sensitivity would be reduced in the PIA reaction. If, however, high titered antisera were used which is type specific, i.e., produced against the ribonucleoprotein, the variations in antigenicity caused by the other antigens would be eliminated and sensitivity would be increased.

It is also recommended that a study be initiated to develop a method for detection of variations in one of the other influenza antigens mentioned, the neuraminidase antigen. This would demonstrate feasibility to determine when there is need for a new flu vaccine to be produced. Present methods involve much time in isolating and characterizing new influenza antigen combinations which appear. If it were possible to test new influenza isolates with a bank of beads sensitized with antibody to various antigenic types, changes could be rapidly determined and new vaccines produced. This would

be very beneficial since, with modern travel, flu can spread very quickly.

In the area of sample handling an unexpected result occurred. When an oropharyngeal wash was tested for PIA reactivity using beads sensitized with adenovirus group antibody, negative samples demonstrated a minor increase in agglutination; however, samples to which adenovirus was added showed a reduction of agglutination when compared to a positive control sample in the usual buffer. Cysteine seemed to reduce the non-specific agglutination but had no effect on increasing the specific agglutination to the level of the positive control. Continued efforts must be given to resolve this problem.

In summary, it is recommended that efforts be continued using the group antigen approach for viral and bacterial detection and in the area of sample handling. Also, optimum conditions for the adenovirus group antigen system should be determined for use in the PIA test.



